LABORATORY MANUAL

PRACTICAL VETERINARY BIOCHEMISTRY

VPB- Unit-I & III

Course Title – General and Analytical Veterinary Biochemistry

(MSVE-2016)

Name – .....

Roll No / Enroll No -...../.....

Batch -.....Session –.....

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FOREWORD

I am glad to see the Laboratory Manual of ***General and Analytical Veterinary Biochemistry****. It am happy to note that the manual covers the practical syllabus of B.V.Sc. & A.H. course as per the standards laid down by Veterinary Council of India.*

Professors previously worked here have prepared this manual utilising their excellent knowledge and expertise in the field of biochemistry. They have covered all the aspects like objectives, outline and description, material and methods and observation to be taken care off.

Definitely this manual will be helpful for smooth and effective conduct of practicals and ensure a handbook for students for entire life in the profession.

Dean

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PREFACE

This Laboratory Manual has been prepared for the undergraduate students of B.V.Sc & A.H.in accordance with the syllabus designed by the Veterinary Council of India. The efforts have been made to make the manuscript worthy, realistic and easily understandable for the students, teachers and Veterinary Practitioners for diagnosis of different microbial diseases of animals. We hope this manual will serve very useful tool to the under graduate and graduate students of Veterinary Science who are undergoing courses in veterinary biochemistry.

It's our pleasure to thank Dean Sir, M.J.F College of veterinary and Animal Sciences, Chomu, Jaipur for providing necessary facilities and rendering all helps in preparing this course manual.

CourseIncharge

Department of Veterinary Physiology &Biochemistrty

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Unit-I

General Veterinary Biochemistry

GENERAL INSTRUCTIONS

- Use apron and other devices like gloves, goggles, mask and lead shield/apron etc. depending upon the material to be handled.
- Do not add water to acids always add acids to water for making solutions. Keep acids off skin; wash off immediately with tap water.
- If acid fall on others, neutralize the same with few drops of dilute ammonia solution or some other weak alkali solution.
- If acid spills on the floor or on the table, neutralize the same with few drops of dilute ammonia solution or some other weak alkali solution.
- If you happen to suck acid into your mouth during pipetting, wash your mouth quickly with water and rinse with a weak solution of washing soda.
- Use fume hood to protect against any type of fumes.
- Glass apparatus to be used should be neat, clean anddry.

CLEANING LABORATORY GLASS WARES

The glass and porcelain wares should be thoroughly washed with somedetergent;then these should be extensively rinsed with tap water followed by further rinsing withdistilledwater.Ifagreasefilmremainsaftercleaningwithdetergent,acleaningsolutionconsistingof sodium or potassium dichromate in concentrated sulphuric acid may be used. After thisrinsingisnecessaryinordertoremovethetracesofdichromateionswhichadherestronglytoglassor porcelainsurface.Lastwashingshouldbewithnormalsalinewhileworkingwithblood.Dryall glassware before use in the oven.

PREPARATION OF CLEANING SOLUTION

Mix 10 to 15 gm potassium dichromate with about 15 ml of water in a 500 ml conical flask. Add concentrated sulphuric acid slowly and stir to 500 ml mark.

Note:

1. Cleaning solution should be discarded when it acquired green colour of chromiumions.
2. Spillage, if occurs, should be cleaned withwater.

INTRODUCTION TO LABORATORY EQUIPMENTS / INSTRUMENTS

1. **Beaker-** A round cylindrical flat bottom laboratory vessel, usually with pouring spout (beak) of a capacityrangingfrom50to1000ofmilliliter.Itisusedforstoring,mixingandheatingsubstances.
2. **Water bath-** Round or rectangular vessel with electric heating element used to boil the solution up to 100^0C.
3. **Bunsen burner-** These are gas burner (gas taps) which uses combustible petroleum gasesas fuel. It is used for heatingsolution.
4. **Burette-** A glass tube with a capacity of the order of 25 to 100 ml and graduations usuallyof 0.05 to 0.1 ml with stopcock attachment used to deliver an accurate measured quantity of liquid.
5. **Burette stand-** It holds the burette vertically duringtitration.
6. **Centrifuge machine-** A laboratory device for subjecting a substance in solution to rotateat extremely high speed to cause deposition of solids (sedimentation) insolution.
7. **Cuvette-** It is small flat bottom glass tube, used to keep the solution into the Colorimeter/ Spectrophotometer for optical densitymeasurement.
8. **Distillation apparatus-** It is an equipment used for the preparation of Distilledwater
9. **Distilled water bottle / Washing bottle-** This is plastic bottle with long nozzle used for easy dispensing ofwater.
10. **Flasks-** It is a laboratory vessel usually made up of glass with constrictedneck.

(a) **Volumetric flask-** It is flat bottom flask with long narrow neck, fitted with an air tight glass stopper. Neck is calibrated to contain or deliver exact volume.

(b) **Conical flask-** It is a conical shaped flask which is used for carrying outtitration.

(c) **Round bottom flask-** It is a glass flask having a round bottom and a long narrow neck. It is used in the experiments where reaction requires heating ofsubstance.

(d) **Flat bottom flask-** It is glass bottle having a round body with slightly flat at the center and long narrow neck. It is used for storing solution and carrying out reactions incold.

11. **Folin Wu tube/ Potato tube-** It is glass tube which is constricted at one end and this constricted part is attached with a round body bulb. It is used for the estimation of bloodglucose.
12. **Funnel-** It is cone shaped wide glass or plastic tube drawn into a long narrow neck. It is used for filtration purpose or pouring liquids in narrow mouthedbottles.
13. **Glass rod-** It is cylindrical thin solid stick of glass. It is used for stirring solutions or mixing of substances.

14. **Measuringcylinder-**Itisgraduatedglasscylindricallaboratorydeviceusedforthe measurement of volume of liquids. It is usually available in the capacity of 50ml to 2 liters graduations for measuring exactvolume.
15. **Ostwald's viscometer-** It is _U' shaped long narrow glass tubes with two bulbs on both the arm of the tube. It is used for the determination of viscosity of different liquids like Benzene, Chloroformetc.
16. **Electronic balance-** It is a balance surrounded by a wall glasses. It is used to measure theweight solids reagents up tomg.
17. **Pipette–**

(a) **Volumetric Pipette / Bulb pipette-** It allows extremely accurate measurement of a volume of solution. These pipettes have a large bulb with a long narrow portion above with a single graduation mark as it is calibrated for a single volume. Volumetric pipettes are commonlyused in analytical chemistry to make laboratory solutions from a base stock as well as prepare solutions fortitration.

(b) **Measuring Pipette-** it is heavy walled long narrow glass tube of small bore with slightlybeveled (Pointed) end used to draw up accurate measurement of definite volume ofsolutions.

(c) **Pasture pipette-** It is long glass tube with narrow constricted part on one end and rubberbulb (dropper) on other end. It is used to transfer the liquid reagents indrops.

18. **Pipette stand-** It is made of a plastic and used for keeping the pipette in laboratory inhorizontal position.
19. **Reagent bottle-** These are cylindrical glass bottles of different shape and size (wide mouthed, small mouthed, dropper bottles etc) and are used for keeping reagents prepared inlaboratory.
20. **Spirit lamp**- It consists of a base vessel storing combustible liquid (spirit) and bears a holefor wick holder. It is not used for strongheating.
21. **Test tube-** It is cylindrical glass tube closed at one end. It is used for carrying out varioustests.
22. **Test tube basket-** It is made up of iron sheet or wire meshwork or plastic used for keeping test tubes after washing andcleaning.
23. **Test tube holder-** It is device for holding the test tube during heating. It is made of metal strip or thickwire.
24. **Test tube stand-** It is made of plastic, wood or iron used for holding test tubes. There are pegs fitted on stand are used for keeping the clean test tube upside down todrain.

25. **Tripodstandwithwiregauze-**Tripodstandismadeofcastironandisusedassupportforheating beakers, porcelain discs etc. The wire gauze is made of fine iron wires fitted with thin asbestos sheet in the center. The beaker / glassware are placed over wire gauze on tripod stand. It helps in heating the glassware evenly thus reduces the chances ofbreakage.
26. **Watch glass-** It is arch shaped glassware used for keeping solid substance in smallquantity.
27. **Spectrophotometer-** It measures either the amount of light reflected from a sample object or the amount of light that is absorbed by the sample object. A spectrophotometer is commonly usedfor the measurement of transmittance or reflectance of solutions, transparent or opaquesolids.
28. **Colorimeter-** It measures the absorbance of particular wavelengths of light by a specific solution or optical density of any solution. This device is most commonly used to determine the concentration of a known solute in a given solution by the application of the Beer-Lambert law, which states that the concentration of a solute is proportional to theabsorbance.
29. **Digital pH meter-** It is an electronic device used for measuring the pH (acidity or alkalinity) of a liquid (though special probes are sometimes used to measure the pH of semi-solidsubstances).
30. **Hot air oven-** These are electrical devices used in sterilization. The oven uses dry heatto sterilize articles. Generally, they can be operated from 50 to 300 °C (122 to 572°F).
31. **Laboratory spatulas-** These are small stainless steel or glass utensils, used forscraping, transferring or applying powders and paste likechemicals.

Exercise No. - 1

Concentration of Solutions and System International (S.I.) Units

Aim

- To know the various common methods of expressing the concentration of asolution.
- To know about the *SystemeInternationaled'Unites*for the various biochemical parametersused in biochemicalestimations.

Concentration of Solutions

- Solutionsmayberegardedasmixturesofsubstances*viz.*thesoluteandthesolvent.Thesubstance that is dissolved is _Solute' and the medium that dissolves the solute is called _Solvent'. The particle size of the solute in solvent is < 1nm.
- The concentration of the solution refers to the amount of the solute present in the given quantity of the solvent orsolution.
- The relative concentrations of the substances in a solution can be measured in severalways:
  - Percentage Concentration(%)
  - Parts per million(ppm)
  - Molarity(M)
  - Molality(m)
  - Normality(N)

Percentage Concentration (%)

- Thisrepresentspartsper100i.e.Numberofpartsofthesolutepresentin100partsofthesolution. Itisdenotedby%.TherearethreewaysofexpressingPercentageConcentration(%)–W/V,W/W and V/V.
- Examples
  - W/V (Weight / Volume): To prepare 9% Saline solution W/V, weigh 9 grams of NaCland dissolve in 100 ml of distilledwater.
  - W/W (Weight / Weight): To prepare 5 % Sugar solution W/W, weigh 5 grams of Glucoseand dissolve in 100 grams of distilledwater.
  - V/V (Volume / Volume): To prepare 25% ethanol, take 25 ml of ethanol and add 75 ml of distilled water, making the total volume to 100ml.

Parts per million (ppm)

- This refers to the number of parts of the solute present in 1 million (10 lakhs) parts ofthe solution. It is denoted asppm.
- Example: 10 ppm chlorine means 10µg of chlorine in 1 g of water. i.e. 1 ppm = 1µg/g or1µg/ml or 1mg/Litre.

Molarity (M)

- It is defined as the number of moles of the solute dissolved per litre of the solution. It is denoted by M; Units: moles / L (smaller unitsmM / L, µM / Letc.)
  - M= Gram molecular weight of the solute / Litre of thesolution

  - The Molecular Weight of NaCl is 58.5. To prepare 1 M NaCl solution, weigh 1 g molecular weight of NaCl i.e. 58.5 g and dissolve in distilled water to make a final total volume of 1litre.

Molality (m)

- It is defined as the number of moles of the solute dissolved per Kg of the solvent. It is denoted by m; Units: moles / Kg (smaller unitsmM / Kg, µM / Kgetc.)
  - M= Gram molecular weight of the solute / Kg of thesolvent
- Example:
  - The Molecular Weight of NaCl is 58.5. To prepare 1 m NaCl solution, weigh 1 gmolecular weight of NaCl i.e. 58.5 g and dissolve in 1000 grams or 1 Kg of distilled water.

Normality (N)

- It is defined as the number of equivalent weight of the solute dissolved per litre of the solution. It is denoted by N; Units: Eq/ L (smaller units mEq / L, µEq / L etc.)
  - N= Gram equivalent weight of the solute / Litre of thesolution
- One-gram equivalent weight of an element or a compound represents its capacity to combineor replace One mole of hydrogen. Ingeneral,
  - Eq. Wt = Molecular Weight / Positive (_+‘) Valences of the Constituentions

1 Mole Eq. Wt=$\frac{\text{1 Mole Ionised Substance}}{\text{Valency}}$

- Example:
  - The molecular weight of Sodium Carbonate (Na_2CO_3) is 106 and equivalent weight is 53. To prepare1NNa_2CO_3solution,weigh53gofitanddissolvein1000mlor1Litreofdistilledwater.

- Semi- Normal Solution: A solution 1 Litre of which contains ½ gm equivalent of solute iscalled as Semi- Normal Solution and denoted byN/2.
- Deci-Normal Solution: 1/10 gm equivalent of substance present in one Litre of a solutionis called Deci-Normal Solution and denoted byN/10.

Osmoles-

- The amount of osmotically active particles is usually expressed inosmoles.
- 1 Osmole = molecular weight of substance (in grams) / no. of freely moving particlesliberated by each molecule in thesolution.

SYSTEME INTERNATIONAL UNITS

- The *SystemeInternationaled'Unites*(SI) or the International system of Units was recommended for use in the health profession by the world Health assembly in May 1977. The SI is the culmination of more than a century of effort to develop universally acceptable units ofmeasure.
- The user of SI has been rapidly gaining acceptance with many nations now mandating its use and many others strongly recommending its use. Furthermore, many scientific journals now require thatunitsbeexpressedinSIalongwiththeconventionalunitsifused.Thefollowingtablegivesa partial list of biochemical parameters with their conventional ―old‖ units and new SI units with conversionfactor:

SI Conversion Factors

S.No.	Biochemical Constituent	Conventional "old" unit	X Factor	= New SI Unit
1.	Albumin	g / dl	10.0	mmol / l
2.	Bilirubin	mg / dl	17.10	µmol / l
3.	Calcium	mg / dl	0.2495	mmol / l
4.	Chloride	mEq / l	1.0	mmol / l
5.	Cholesterol	mg / dl	0.02586	mmol / l
6.	Creatinine	mg / dl	88.4	µmol / l
7.	Fibrinogen	mg / dl	0.01	g / l
8.	Globulin	g / dl	10.0	mmol / l
9.	Glucose	mg / dl	0.05551	mmol / l
10.	Phosphorous	mg / dl	0.3229	mmol / l
11.	Potassium	mEq / l	1.0	mmol / l
12.	Protein	g / dl	10.0	mmol / l
13.	Triglycerides	mg / dl	0.01129	mmol / l
14.	Urea	mg / dl	0.1665	mmol / l
15.	Urea Nitrogen (BUN)	mg / dl	0.3570	mmol Urea / l
16.	Uric acid	mg / dl	59.48	µmol / l
17.	*Enzymes	U / l	16.67	nkat / l

* There is yet no general agreement or recommendation for the use of the katal (1 kat = 1 mol / s) in place of the widely used international unit (1 U = 1 µ mol / m). The U / L should be continued to be used for all enzyme activities.

Exercise No. - 2

Preparation / Standardization of Acids / Alkali

a. **A. Standardization ofAlkali**

Aim

- To prepare 1000 ml each of 0.1 N Oxalic acid and 0.1 N NaOH (approx.)solutions.
- To determine the strength of the above NaOH solution using standard 0.1 N Oxalic acidsolution.

Titration

- This is the experimental technique by means of which we measure the exact volume of one solution, which is required to react with given volume of another. Practically titration is usually performed by adding a solution of known solute concentration to another solution of unknown concentration, until the desired reaction is completed. In the titration of an acid with a base, a dye (indicator)maybeaddedwhichhasapropertyofexhibitingonecolourinacidandanothercolour in the basic solution As soon as sufficient base has been added to neutralize it. Further additionof basic solution causes the dye/ indicator to change thecolour.
- The neutralization reaction that occurs between soluble acids and soluble bases is usually rapid and quantitative. The reaction is completed when the no. of equivalents of an acid is equal to equivalents ofbase.

  $\frac{\text{Wt of acid}}{\text{Eq .wt. of acid}} = \frac{\text{Wt of base}}{\text{Eq. wt. of base}}$

  $\mathbf{N_1V_1 = N_2V_2}$

End point

- In an acid alkali titration, end point is that point at which the titration is stopped being shown by the colour change of the particular indicator used. Indicator depends upon the pH value at which the indicator shows its specific colourchange.

Principle

- One equivalent of an acid furnishes one mole of hydrogen ions (H), and one equivalent of a base furnishes one mole of hydroxide ion, (OH). One equivalent of an acid reacts with one equivalent of a base. If you know the number of equivalents of an acid in a given volume of a solution,you

can find the volume of a base i.e. chemically equivalent to this volume of the acid solution, than that volume of base contain the same number of equivalents. Therefore you can easily calculate the concentration in terms of equivalent per liter of the solution of the solution the base. To standardize a solution of sodium hydroxide, oxalic acid dihydrate $H_2C_2O_42H_2O$, is used as a primary standard acid. A primary standard acid is a solid whose mass is an accurate measure of thenumberoftheequivalentitwillfurnish.Thenetequationofneutralizationofreactionspecifies that there are low equivalents of oxalic acid permole.

$$H_2C_2O_4 .2H_2O+2NaOH \longrightarrow Na_2C_2O_4+2H_2O$$

Requirement

- Pipette
- Burette
- Conicalflasks

Theory

- Strength of the solutions to be prepared is in Normality,wherein

Normality (N) = It is the number of gram equivalent weight of the solute dissolved in one litre of the solution.

Preparation (Oxalic acid solution)

Preparation of 1000 ml of Standard N/10 Oxalic acid solution

- Oxalic acid is a dibasic acid and available in crystalline form as $(COOH)_2.2\ H_20$. This is taken as primary standard for acids as it can be weighedaccurately.

Equivalent weight of Oxalic acid = (Molecular weight / 2) = (126 / 2) = 63 g

- To prepare 1000 ml of 1 N Oxalic acid solution, the amount of oxalic acid required = 63g.
- Therefore, to prepare 1000 ml of 0.1 N Oxalic acid solution, the amount of oxalic acid required= 6.3 g.

Procedure

- Weigh 6.3 g of Oxalic acid exactly and dissolve it in 1000 ml of distilled water in a beakerand stirwell.

Preparation (NaOH solution)

- Sodium hydroxide is a monoacidic base and is available as pellets, which are hygroscopic. **Equivalent weight of NaOH**= Molecular weight = 40g.

- So, to prepare 1000 ml of 1 N NaOH solution, the amount of NaOH required = 40g.
- Therefore, to prepare 1000 ml of 0.1 N NaOH solution, the amount of NaOH required = 4.0gm.

Procedure

- Weigh exactly 4.0 g of NaOH and dissolve it in 1000 ml of distilled water in a beaker andstir well.

TITRATION

- Pipette out 5 ml of standard Oxalic acid solution into a conicalflask.
- Add two drops of Phenolphthalein indicator and mix well (it will be colorless).
- Fill the burette with NaOH solution and note the initialreading.
- Then add the NaOH solution drop by drop from burette to the conical flask until a pale permanent pink color appears and note the finalreading.

Observation

$(COOH)_2$vs. NaOH (Indicator: Phenolphthalein)

S.No.	Volume of Standard $(COOH)_2$ in ml	Burette Reading ml		Volume of NaOH Consumed in ml	Concordant Value in ml
		Initial	Final		

Calculation

By Normality equation, $\mathbf{V_1N_1 = V_2N_2}$

N_1 = Normality of $(COOH)_2$= 0.1 N

V_1 = Volume of the $(COOH)_2$ = 5 ml

N_2 = Normality of NaOH= ? (Unknown)

V_2 = Volume of NaOH (from observation)

Substituting the values in the above equation, We get,

Result

The strength of NaOH solutionprepared is_____N.

Precautions

- Burette and pipette should be rinsed with the respectivesolutions.
- Approximately same quantity of indicator should be used for eachtitration.

B. Standardization of Acid

Aim

- To prepare 1000 ml each of 0.1 N HCl (approx.) and 0.1 N Na_2CO_3solutions.
- To determine the strength of the above HCl solution using standard 0.1 N Na_2CO_3 solution.

Normal solution: Normal solution contains 1gm of ionisable replaceable hydrogen or its equivalents in 1 litre ofsolution.

To make Normal Solution with a liquid, formula is:

$$\textbf{Desired value of liquid(ml/L)} = \frac{\textbf{Molecular Weight}}{\textbf{Valency X Specific gravity X \% Concentration}}$$

Example:-

1. To make Normal solution ofHCl

Mol. Wt of HCl = 36.5, Valency = 1, Concentration of the solution = 36%, Specific gravity = 1.19

So Desired volume of 36% HCl to from 1L normal solution $V = \frac{36.5}{1X1.19X0.36}$

$V = 85.2$ ml/L

- Place 500 ml of distilled water in 1Lvolumetric flask, then take 82.2 ml concentrated HCL and mix the content properly. Finally make the final volume 1000 ml or 1Liter by adding distilled water up to themark.
- To prepare 0.1N HCl, take 8.52 ml of concentrated HCl and make final volume to 1L byadding distilled water in 1000 ml or 1L volumetricflask.

2. To make normal solution ofH_2SO_4

Mol. wt of H_2SO_4= 98, Valency = 2, Concentration of the solution = 96%,

Specific gravity = 1.83

So Desired volume of 96% H_2SO_4 to from 1L normal solution $V = \frac{98}{2X1.83X0.96}$

$V = 27.8$ ml/L

Requirement

- Pipette
- Burette
- Conicalflasks

Theory

- Strength of the solutions to be prepared is in Normality, wherein

- **Normality (N)** = It is the number of gram equivalent weight of the solute dissolved in one litre of thesolution.

Preparation of 1000 ml of N/10 HCl Acid Solution

- HCl is a monobasic acid and is aqueousanhydrous.

  Equivalent weight of HCl = Molecular weight / 1 = (1+35.5) / 1 = 36.5 / 1 = 36.5 = 36 g (Approx)

  Specific gravity (of the commercially available HCl) = 1.18 (1.18 g of HCl / 1000 ml) = 1180 g of HCl / 1000 ml
- We know that 36 g of HCl in 1000 ml = 1N
- Then, 1180 g of HCl in 1000 ml will be = 1 x 1180 N /36 = 32.78N
- But the assay (purity) of the commercially available HCI solution =36%
- Therefore, the actual normality will be = (36/100) x 32.78=11.79N
- We are required to prepare 1000 ml of 0.1N HCl using this above 11.79 N HCl stocksolution.
- Therefore, the Volume of Stock (Conc.) HCl required can be calculated by using theequation:

$$V_1N_1 = V_2N_2$$

  - Where, N_1 = Normality of the Conc. HCl = 11.79N
  - V_1 = Volume of the Conc.HCl(Unknown)
  - N_2 = Normality of HCl to be prepared = 0.1 N
  - V_2 = Volume of HCl to be prepared = 1000ml
- Substituting the Values, we get 11.79 N x V1 = 0.1 N x 1000ml
- Then, V_1= ((0.1 N x 1000 ml)/11.79N)=8.48ml
- Therefore, 8.48 ml of HCl is diluted with distilled water to make up the volume to 1000ml.

Procedure

- The Commercially available Conc. HCl is approximately 11.79N.
- Take 8.4 ml of the above HCl and initially dilute it by slowly adding drop by drop and stirringit in about 500 ml of distilledwater.
- Then make up the volume to 1000 ml in distilledwater.

Preparation of 1000 ml of Standard N/10 Na_2CO_3 Solution

- Na_2CO_3 is used as primary standard for alkali because it is obtained in pure state and weighed accurately.

Equivalent weight of Na_2CO_3 = ((23 x 2 + 12 + 16 x 3) / 2)= 53 g

- So to prepare 1000 ml of 1 N Na_2CO_3 solution, amount of Na_2CO_3 required will be 53 g and for 1000 ml of 0.1 N Na_2CO_3 solution the amount of Na_2CO_3 required will be 5.3g

Procedure

- Weigh exactly 5.3 g ofNa_2CO_3.
- Then transfer this Na_2CO_3 to a beaker containing about 800 ml of distilledwater.
- Dissolve the Na_2CO_3 completely bystirring.
- Add more distilled water and make up the volume to 1000ml.

Titration of HCl with Standard 0.1 N Na_2CO_3

- Pipette out 5 ml of standard N/10 Na_2CO_3 solution into a conicalflask.
- Add two drops of methyl orange indicator and mix well (a pale yellow color willdevelop).
- Fill the burette with HCl solution and note the initialreading.
- Then add the HCl solution drop by drop from burette to the conical flask until the colorchanges from orange to slight pink and note the finalreading.
- Repeat the process until consecutive readings areobtained.

Observation

- Na_2CO_3 vs. HCl (Indicator: MethylOrange)

SI.No.	Volume of Standard Na_2CO_3 in ml	Burette Reading ml		Volume of HCLConsumed in Ml	Concordant Value in ml
		Initial	Final		

Calculation

- By Normality equation, $\mathbf{V_1N_1 = V_2N_2}$
  - N_1 = Normality of Na_2CO_3 = 0.1N
  - V_1 = Volume of the Na_2CO_3 = 5ml
  - N_2 = Normality of HCl =?(Unknown)
  - V_2 = Volume of HCl (fromobservation)

Result

- The strength of HCl solutionpreparedis_____N.

Precautions

- Burette and pipette should be rinsed with the respectivesolutions.
- Approximately same quantity of indicator should be used for eachtitration.

Questions

1. A bottle of HCI with following specifications is given toyou.
Specific gravity =1.18
Purity = 35%
Desired volume to make Normal solution of HCL?
Hint: To make Normal solution of liquid- By Specific gravity Method

$$ML/Lt = \frac{\text{MolecularWeight}}{\text{Valency X Specific gravity X Percent Concentration}}$$

2. A bottle of H_2SO_4 with following specifications is given to you.
Specific gravity =1.835
Purity = 98%
How will you prepare 4 N solution of H_2SO_4?

Exercise No. - 3

Preparation of Buffers

Principle

A buffer solution is one that can resist change in its pH on the addition of an acid or base. Most of the buffer solution consists of mixture of either weak acids and their salts or weak base and their salts. Buffer solutions possess reserve acidity as well as alkalinity due to the presenceof $CH_3\ COO^-$ ions. If an acid is added to this solution, the H^+ ions furnished by the acid combine with acetate ions of form un-dissociated molecules of acetic acid.

$$CH_3\ COO^- + H \longrightarrow CH_3COOH$$

Thus the H+ ion activity (pH value of the ammonium acetate solution changes slightly upon addition of acid when a base is added to ammonium acetate solution) the base will reduce the effective concentration of H^+ ions.

$$H^+ + OH^- \longleftrightarrow H_2O$$

The resulting loss in H^+ ion is compensated by the reaction:-

$$NH_4^+ \longrightarrow NH_3 + H^+$$

Thus the buffer solution can resist a change in pH upon addition of an acid or a base.

Buffering capacity

Howmuchacidorbaseabufferwilltoleratewithoutchangingitsp Hiscalledthebuffering capacity. This will depend upon the concentration of the buffer. Thus 0.01 M buffer will have ten timeslowbufferingcapacitythana0.1MBufferconcentrationisthesumtotaloftheconcentration of two bufferingcomponents.

Criteria for selection of buffers

An ideal Buffer should:-

1. Have adequate buffering capacity in required pH range. Ex. Tris- buffer (pKa= 8.3) has apH range of 7 to9.
2. Be chemically inert and should not react or bind with bio-molecules or other components particularly for assaying activities of enzyme involving metalions.
3. Be available in high degree ofpurity.
4. Be enzymatically and hydrolyticallystable.
5. Maintain pH, which is minimally influenced by temperature ionic composition andconcentration or salt effect ofmedium.
6. Not absorb light in the visible or UV-region ofspectrum.
7. Be non-toxic.

Composition of some commonly used Buffer

Buffer can be made in stock solutions and these are diluted before use.

1. Phosphate buffer (Sorenson'sbuffer)

pH range : 5.8 - 8

Advantage: Most physiologically common buffer.

- Non-toxic tocells.
- Stable to several weak at 4^{o}

c. Disadvantage:

- Precipitate more likely to occur during fixation, tends to form precipitate in presence of Ca^{+2}Ion.
- Become slowly contaminated by micro-organisms. Preparation of Buffer(stock):

A. 0.2M dibasic sodium phosphate =12

$Na_2HPO_4 .2H_2O$ (Mol. wt = 178.05 gms) = 35.61 gms

or $Na_2HPO_4.7H_2O$ (Mol. wt = 268.07gms) =

53.65gms or $Na_2HPO_4 .12H_2O$ (Mol. wt = 358.14 gms) =

71.64 gms +ddH_2O, to make 1 lt.

B. 0.2M monobasic sodium phosphate =1L

$Na_2HPO_4 .H_2O$ (Mol. wt = 138.01 gms) = 27.6 gms

or $Na_2HPO_4. 2H_2O$ (Mol. wt = 156.03gms) = 31.21gms.

+ ddH_2O, to make 1 lt.

Buffer solution (Working):

Mix _x' ml of 0.2M dibasic solution with _y' ml of 0.2M monobasic solution. Dilute to 100 ml with ddH_2o.

2. Veronal - Acetate buffer (MichaelisBuffer)

Advantage: Useful for block staining with uranyl acetate since precipitation do not from.

Disadvantage:

- Reacts withaldehydes.
- Buffer poor at physiological pH.
- Supports growth ofmicro-organism
- ContainsBarbiturate

Preparation of buffer (stock): 0.28M (100 ml)

A. Sodium -veronal (Barbitone- Sodium) = $C_8H_{11}O_3N_2Na$ (Mol. wt =206.68)

= 2.89 gms

B. Sodium - acetate (anhydrous) = CH_3CooNa (Mol. wt = 82.03)=1.15gms

or Sodium - acetate (hydrated) = $CH_3CooNa.3H_2O$ (Mol. wt =136.09) =1.90gms

+ H_2O, to make 100 ml.

Buffer (Working solution):

5 ml of veronal acetate stock solution with 15 ml ddH_2O. Add gradually 0.1 HCl to desired pH.

3. Tris-Buffer

Advantage:

- Good buffering capacity at higher pH, required for some tissue and somecyto-chemical processes.
- More or less physiologicallyinert.

Disadvantage: pH changes with temperature must be measured at desired temperature.

A. Tris buffer: - pH range7.1-8.9

Tris (hydroxylmethyl) amino methane (Mol. Wt =121.13) = 24.2gms

+ ddH_2O, to make 1 lt.

Adjust pH of 50ml stock solution with 0.1mNaOH, dilute to 100 ml with ddH_2O.

B. Tris maleate Buffer: pH range5.8-8.2

Tris (hydroxylmethyl) amino methane (Mol. wt = 121.13) = 24.2 gms

+ Maleic acid (Mol. Wt = 116.07) = 23.2gm

+ ddH_2O to make 1 lt.

Working Buffer: 0.2M-100ml.

Adjust pH of 50ml stock solution to desired pH with 0.1m NaOH, dilute to 100 ml with ddH_2O.

Range of common Buffer solutions

S. N.	Buffer	pH range at 25^oC
1.	Citric acid – Na citrate	3-6.2
2.	Na acetate- Acetic acid	3.7 - 5.6
3.	$NaHCO_3$- NaOH	9.6 – 11
4.	Na_2CO_3 - $NaHCO_3$	9.2 - 10.8
5.	Tris (hydroxylmethyl) amino methane-HCl	7 – 9

Material

- Sodium carbonate, sodium bicarbonate, distilled water pH meter, 0.5N HCI, 0.5 N NaOHetc.

Method

- Prepare buffer solutions of different pHvalues.
- Preparation of buffer solution: 0.1M Na_2CO_3 and 0.1 M $NaHCO_3$solutions.
- Using these stock solutions a buffer of known pH value can be made by mixingdifferent amounts of A and B as given in the tableunder:

0.1 M $Na_2 CO_3$	**0.1 M $NaHCO_3$**	**Buffer pH value**
7.0	93.0	9.0
12.0	88.0	9.2
18.0	82.0	9.4
27.0	73.0	9.6
39.0	61.0	9.8
51.0	49.0	10.0
62.0	38.0	10.2
73.0	27.0	10.4
82.0	18.0	10.6
90.0	10.0	10.8
94.5	5.51	11.0

- To 50 ml buffer solution add 150 ml distilled water and then add different quantities of acid and base. Measure change in pH value with the help of a pHmeter.

Observation

0.5 N HCI added (ml)	**Change in pH**	**0.5 NaOH added (ml)**	**Change in pH**
0.2		0.2	
0.2		0.2	
0.2		0.2	
0.2		0.2	
0.2		0.2	
0.2		0.2	

Exercise No. - 4

Titration Curve of Acid verses Base

Aim

- To find out the pKa of an acid.

Principle

- Titration is a procedure in which we measure the quantity of a known reagent required toreact with an unknown sample, from which we deduce the concentration of theunknown.
- The NaOH is added in small increments to a known volume of weak acid till neutralization, as determined by the pHmeter.
- The plot of pH against the volume of NaOH added is called as the _Titration Curve‖. This curve reveals the pKa (negative logarithm of dissociation constant) of anacid.
- Pka value is equal to the pH at which the acid is half titrated. The pH can therefore beobtained from knowledge of the end point of thetitration.

Material

- pH meter
- Burette
- 0.1NCH3COOH
- 0.1 NNaOH

Procedure

Titration of acetic acid vs. sodium hydroxide

- Prepare 100 ml of each 0.1N CH3COOH and O.1NNaOH.
- Take 50 ml of 0.1N CH3COOH in abeaker.
- Measure the initial pH of the 0.1N CH3COOH, using a pHmeter.
- Add 1-2 drops of assignedindicator.
- Add exactly 2 ml of 0.1N NaOH from the burette, mix well and measure thepH.
- Repeat the above procedure till the pH of the solution is about 8–10.
- Plot a graph taking the volume of NaOH added in X-axis and pH values in Y-axis.
- Connect all the points and observe the nature of this titrationcurve.
- Find out the pKa of acetic acid, exactly at the half-saturation point of thetitration.

Result

- The pKa value of acetic acidis _____

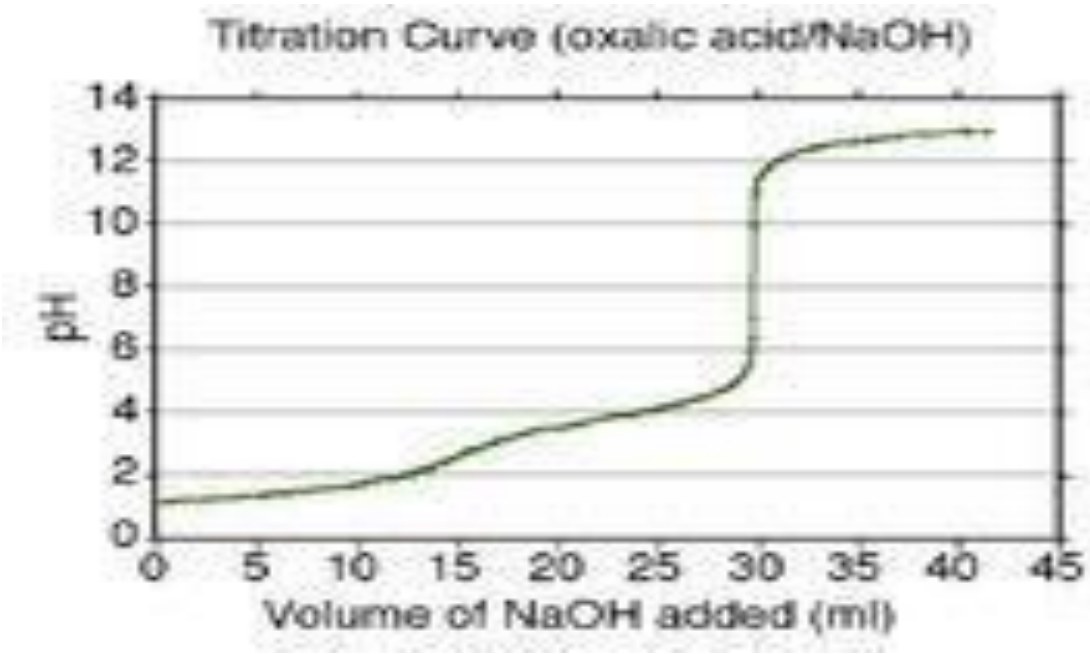
Theoretical Consideration

- At the beginning of the titration, before NaOH is added, very small quantity of acetic acidis ionized.
- As NaOH is gradually introduced, the added OH^- ion combines with the free H^+ ion in the solution to formwater,

$$CH_3COOH \rightarrow CH_3COO^- + H^+$$

$$\mathbf{K_a} = [([H^+]\,[CH_3COO^-])\,/\,[CH_3COOH]] = 1.74 \times 10^{-5}\ M$$

- As free H^+ ions are removed, acetic acid dissociates further to make K_a as constant. As the titration proceeds by the addition NaOH more and more acetic acid ionizes, formingacetate.
- At the midpoint of the titration at which exactly 0.5 equivalent of NaOH has been added, onehalf oftheoriginalCH_3COOHhasundergonedissociationsothattheconcentrationoftheprotondonor (CH_3COOH) now equals that of the proton acceptor (CH_3COO^-). The pH of the equimolar solution of acetic acid and acetate is exactly equal to the pKa of acetic acid(4.76)
- As the titration is continued by the further addition of NaOH, the remaining un-dissociated acetic acid is gradually converted into acetateions.
- The end point of the titration occurs at about 7, in which all the acetic acid has lost its proton to OH^- ions, to form water andacetate.
- The titration curve is used not only to find out the pKa of a weak acid but also shows graphically that a weak acid and its conjugate base can act as abuffer.



Indicator Solutions

- They are weak acids or weak bases whose unionized molecules exhibit one color whereas their anion or cation will have a differentcolor.

S.No.	Indicator	pH Range	Color Change	pKa
1	Methyl Orange	3.0 - 4.4	Red - Yellow	-
2	Methyl Red	4.4 - 6.2	Red - Yellow	5.0
3	Phenolphthalein	8.3 -10.0	Colorless - Red	9.7
4	Bromophenol Blue	3.0 – 4.0	Yellow - Blue	4.0
5	Thymol Blue	1.2 - 2.8	Red - Yellow	1.7
6	Cresol Red	7.2 - 8.8	Yellow - Red	-
7	Phenol Red	6.8 – 8.2	Yellow - Red	7.9

Indicators

An approximate idea of the pH of solution can be obtained using indicators. These are organic compounds of natural or synthetic origin whose colour is depending upon the pH of the solution. Indicators are usually weak acids or weak bases which dissociate in the solution. Disadvantages of pH indicator:

- However, this colour change occurs over a wide pH range so indicators will give onlyan approximate indication ofpH.
- Indicators are affected by oxidizing agents, reducing agents, salt concentration andprotein.
- A final precaution to be taken, when using them is to add only a small quantity of indicator to the solution under examination, otherwise the acid-base equilibrium of the test solution may be displaced and the pH changed.

Exercise No. – 5

Qualitative tests for Carbohydrates and Identification of unknown Carbohydrates

1. Molisch's Test (Principle test ofCarbohydrates)

Principle

The carbohydrate group forms furfural compound when react with concentrated Sulphuric acid. These compounds give deep purple colour with Molisch's reagent. It is a very sensitive test and is given by all carbohydrates. Therefore, this test is performed first to establish the presence of carbohydrate.

Procedure

Take 5 ml of carbohydrate solution in a test tube, to this add 1 drop of Molisch's reagent andthenadd2mlofconcentratedSulphuricacidfromthesidewallsofthetesttubetofromalayer below the carbohydratesolution.

Observations and inference

A purple colour ring is formed at the junction of two layers, indicate the presence of carbohydrate.

2. Iodine Test (Test forPolysaccharides)

Principle

Starch gives blue colour with iodine.

Procedure

Take 5ml carbohydrate solution in a test tube, to this add 2 drops of iodine solution, warm the tube and again cool it observe the change in colour.

Observations and inference

The starch gives blue colour with iodine which disappears on heating and reappears on cooling (glycogen gives red colour with iodine).

3. Reducing Test (Determination of Reducing and Non-Reducing Sugar)

Principle

Reducingsugarshaveabilitytoreducemetalionsinalkalinemedium.InFolinWumethod reducing compound reacts with alkaline copper sulfate and reduces cupric (Cu^{++}) to cuprous (Cu^{+}). Cuprous on reaction with phosphomolybdate gives molybdate blue.

$$CuSO_4 + 2\ NaOH \longrightarrow Cu(OH)_2 + NaSO_4$$

Reducing sugar + $Cu(OH)_2$ + phosphomolybdate ⟶ Molybdate blue

Non reducing sugar + phosphomolybdate ⟶ No colour change

Material

- Folin Wu tubes, boiling water bath, pipettes, alkaline copper sulfate, phosphomolybdate, reducing and non- reducingsugars.

Preparation of Reagents

- **Alkalinecoppersolution**:Dissolve40gofanhydroussodiumcarbonatein about400mlofwater and transfer to one litre volumetric flask. Add 7.5g of tartaric acid, when it gets dissolved, add 4.5g of crystalline copper sulfate. Dissolve and make up to alitre.
- **Phosphomolybdate solution**: To 35g of molybdic acid and 5g of sodium tungstate, add 200 ml of 10% sodium hydroxide and 200 ml of water. Boil vigorously for 30 min to remove nearly all ammoniapresentinmolybedicacid.Coolanddiluteto350ml,nowadd125mlof85%phosphoric acid and dilute to 500ml.

Method

- Take 1 ml of sample in 4 Folin Wu tubes and add 1 ml alkaline coppersulfate.
- Keep the Folin Wu tubes in boiling water bath for 10min.
- Cool these tubes under tapwater.
- Add phosphomolybdate to each Folin Wu.tube.

Observation

Sample	Alkaline copper Sulfate	Phosphomolybdate	Inference
Starch	0.1 ml	1 ml	
Sucrose	0.1 ml	1 ml	
Glucose	0.1 ml	1ml	
Gelatin	0.1 ml	1 ml	

Result

- The above observations show that ---------- is /arenon-reducingand---------- is/are reducing sugar (s)

Precaution

- Pipetting should be done carefully, and air bubble (s) should beavoided.

4. Benedict's test for Distinguishing Reducing and Non-Reducing Sugar

Principle

Carbohydrates may be classified as reducing and non-reducing sugars. Sugars having the aldehydeorketonegroupsarereducingsugarsandsugarsnothavingfreealdehydeorketonegroup arenon-reducingsugars.Benedictreagentisusedfordetectingreducingsugars.Reducingsugars reduce cupric ion (Cu^{2+}) to cuprous ion (Cu^{+}) cuprous is less soluble and form Cu_2O (cuprous oxide) as a red or green or yellow precipitate. The reducing sugar in turn isoxidized.

$$CuSO_4 + 2NaOH \longrightarrow Cu(OH)_2 + NaSO_4$$

$$CHO\text{-}(CHOH)_4 - CH_2OH + Cu(OH)_2 \longrightarrow COOH - (CHOH)_4 - CH_2OH + Cu_2O + H_2O$$

Onic acid Red ppt.

Material

- Test tube, pipette, spirit lamp, test tube holder, samples (Sucrose, glucose, fructose,xylose, lactose) and Benedict reagent.

Preparation of Benedict reagent

- Dissolve173gofsodiumcitrateand100gofsodiumcarbonateinabout800mlofwaterwiththe aid of heat. Dissolve 17.3 g crystalline copper sulfate in 100 ml of water and add slowly to the citrate-carbonate solution. Stirringconstantly.

Method

- Take 2 ml of each sample in test tube and add 1 ml of Benedict'sreagent
- Heat the test tube for a few minutes withshaking.
- Observe the colour, change in the testtube.

5. Fehling's Test For Distinguishing Reducing and Non-Reducing Sugar

Principle

- In this test, the reducing sugars produce a red colored Cu_2Oprecipitate.
- Sodium – potassium tartrate (Rochella salt) present in Fehling's reagent, acts as the chelating agent.

Method

- Mix equal volumes of Fehling's A and B solutions in a test tube and add 1 mL of testsolution.
- Mix well and heat the tube gently over aflame.

- Look for the presence of first green, then yellow & finally red colored cuprous oxideprecipitate.

Observation

- Reducing sugar containing tube shows change in the colour while non reducingsugar containing tube doesnot.

Sample (2 ml)	Benedict reagent	Inference
Xylose	1 ml	
Glucose	1 ml	
Lactose	1 ml	
Fructose	1 ml	
Sucrose	1 ml	

Result

- ---------- is/are reducing sugar(s)and----------is/are non-reducingsugar(s)

Precaution

- During heating, continuous shaking of the tube isnecessary.

6. Seliwanoff's Test (Differentiation of Glucose and Fructose)

Principle

The furanose ring of fructose is more active than pyranose ring of glucose. Therefore, fructose forms furfurals components more quickly.

Procedure

Take 5ml carbohydrate solution in a test tube, to this add 1-2 drops of seliwanoff's reagent and boil it.

Observations and inference

Aredcolourobserveswithin30secondofboilingiffructoseispresent.Thistestisnegative whenglucoseispresentbutsometimesafterprolongedboiling(5-6minutes)theglucosewillgive positivetest.

Seliwanoff's reagent:

Resorcinol – 0.05 gms.+ Conc. Hcl- 33ml and water to make 100 ml.

7. Osazone Test (Differentiation of Maltose and Lactose) Principle

An organic compound known as phenyl hydrazine reacts with carbonyl group of sugar to from the osazone. These osazones have yellow colour and characteristic crystalline form. These are glucosazone, fructosazone and lactosazone. The glucose and fructose gives identical needle shaped osazones.

Procedure

Take 7-8 ml of carbohydrate solution in a test tube and to this add pinch of phenyl hydrazine and double the quantity of sodium acetate add 10 drops of acetic acid. Dissolve by shaking and warming boil the solution for 30 minutes is boiling water bath and allow cooling slowly. Then observe the shape of crystals under the low power microscope.

Observations and inference

Lactose forms power-puff shaped crystals and maltose forms sunflower shaped crystals while glucose and fructose forms identical needle shaped crystals.

8. Barfoed's test (Differentiation of MonosaccharidesAnd Disaccharides) Principle

The Barfoed's test is to distinguish between the monosaccharides and disaccharides. A differential rate of reaction with cupric acetate in acetic acid forms the basis of the test

$$\underset{\text{D-Glucose}}{CHO-(CHOH)_4-CH_2OH} + 2\underset{\text{Cupricacetate}}{Cu(CH_3COO)_2} + 2H_2O \rightarrow \underset{\text{Gluconic acid}}{COOH-(CHOH)_4CH_2OH} + \underset{\text{Curprous Oxide}}{Cu_2O} + 4CH_3COOH$$

The rate of reaction is determined by the speed of formation of Cu_2O. Equimolar concentrations of monosaccharides and disaccharides having one reducing group each per molecule react differently with the Barfoed's reagent. One difference between monosaccharides anddisaccharidesismolecularsize,andthisappearstobelimitingfactorintherateofreaction.It would seem that the smaller molecule has a greater reactivity; the monosaccharides will usually react within 2-5 minutes, while the disaccharide will react within 7-12minutes.

Material

- Test tube, boiling water bath, pipette, sample and Barfoed‘sreagent.

Preparations of reagent

- Powdered copper acetate, 66.5 grams is dissolved in 1 litre of distilled water, filter the solution through Whatman filter paper No 1 and add 25 ml of 36% acetic acid (9 mlglacial acetic acid diluted to 25 ml with distilledwater).

Method

- Take 4 ml Barfoed‘s reagent in testtube.
- Add 1 ml. Sample in the testtube.
- Keep the test tube in boiling water bath for about 5min.

Observation

Sample	Barfoed’s reagent	Inference
Glucose	4 ml	
Fructose	4 ml	
Xylose	4 ml	
Sucrose	4 ml	

Result

- ---------- is/ are monosaccharide(s)and ---------- is/are disaccharide(s)

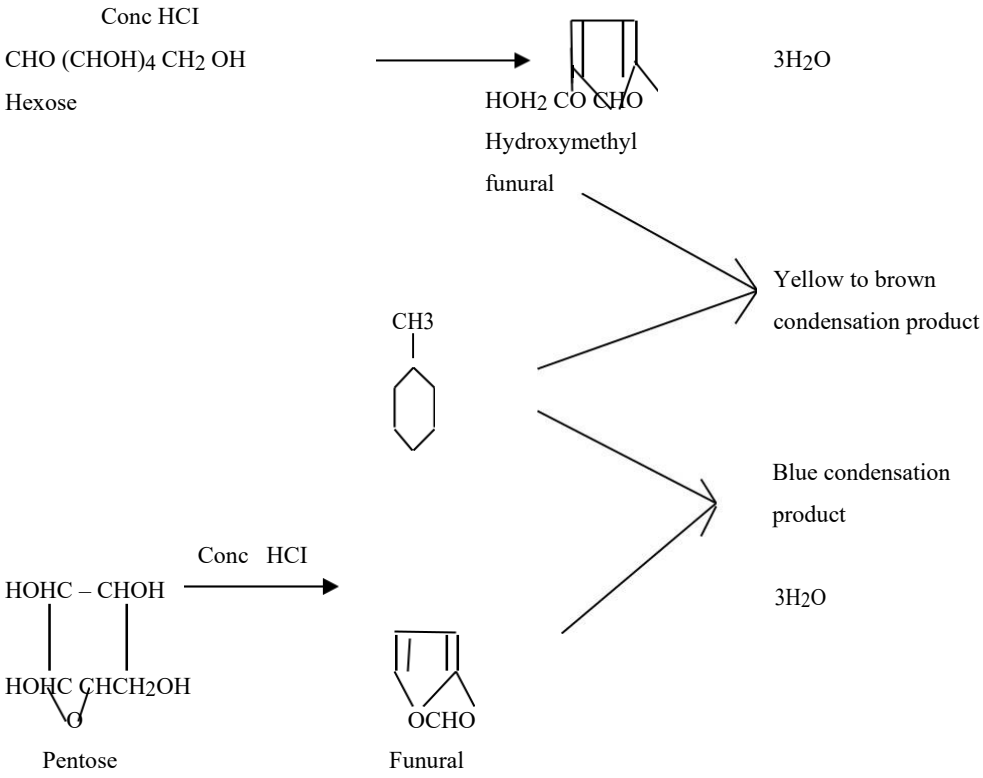
Precautions

- The test tube should not be directlyheated.
- Prolonged heating should because it may produce red precipitated even in theabsence of monosaccharide (s) due to hydrolysis of disaccharide(s).

9. Bial’s Test for Pentose Sugar

Principle

Bial‘s ordinal reaction is used for the determination of pentoses and nucleotides, which contain pentose sugar. The reaction is not specific for pentoses since 2- deoxypentoses, 6-deoxy pentoses,triosesandcertainheptosesproduceidenticalbrightbluecolor.Hexosesgiveyellowthe basis of this test differential production of furfural when either product condenses with orcinal a blue condensation product is formed with furfural and a yellow condensation product is formed with hydroxymethyl furfural the condensation product is soluble in amylalcohol.



Material

- Test tube, spirit lamp sample and Bial‘s reagent

Preparation of reagent

- Add 10 ml of HCI to 2 ml fresh 2% aqueous orcinol and dilute to 18 ml with distilledwater.

Method

- Take 2 ml Bial‘s reagent in a test tube
- Add 1 ml sample to it and heat fewminutes

Observations

Sample	Bial’s reagent	Inference
Xylose	1 ml	
Arbinose	1 ml	
Glucose	1 ml	
Sucrose	1 ml	
Lactose	1ml	

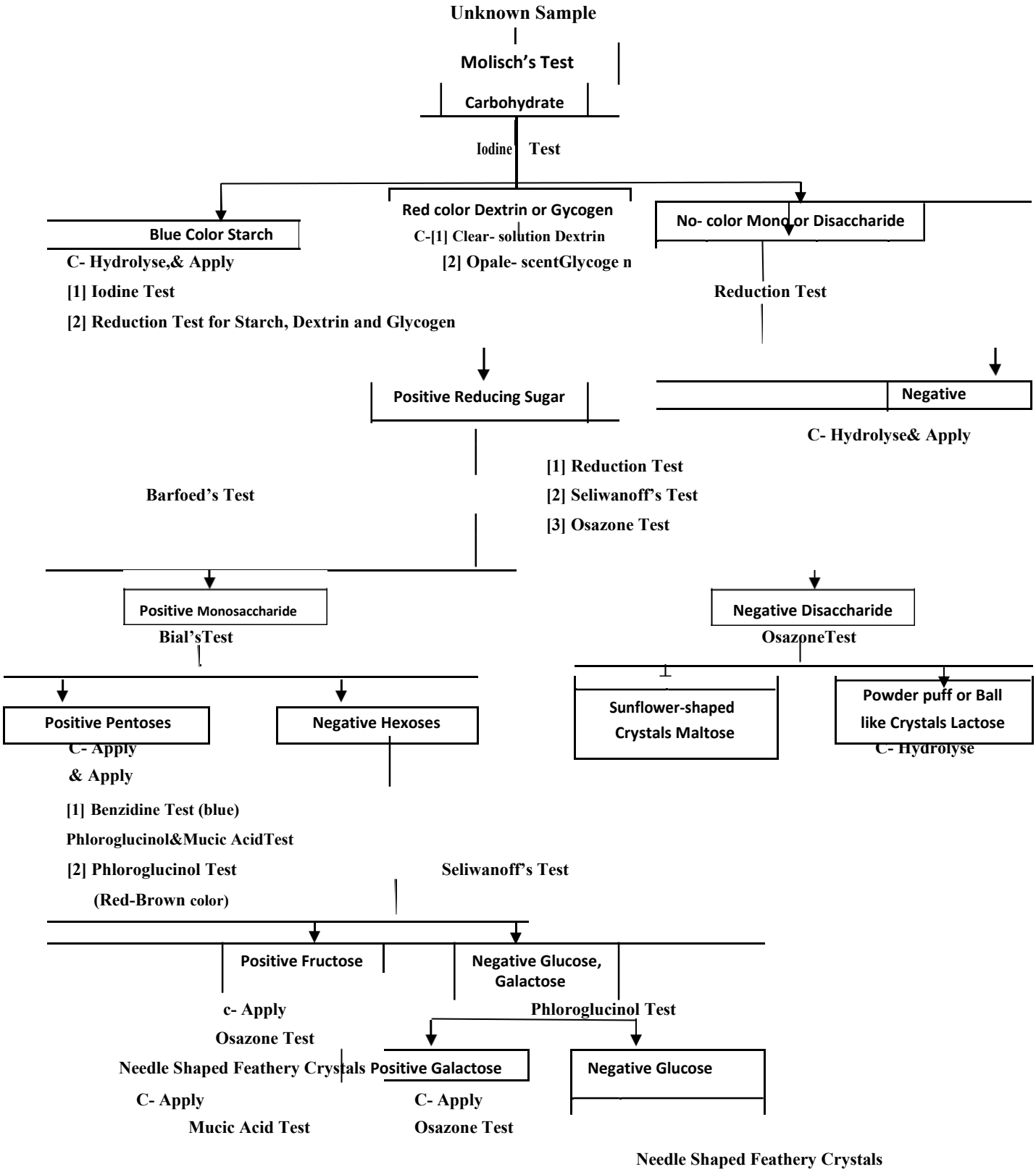
Result

- ---------- is/are pentose sugar (s) and----------is/are ---------- not pentose sugar(s)

Precautions

- Keep shaking the test tube duringboiling
- Heat test tube till sampleboils.

SCHEME FOR IDENTIFICATION OF AN UNKNOWN CARBOHYDRATE



Exercise No. - 6

Determination of Acid Number of an Oil

Definition

- Acid number is defined as ―the number of milligrams of 0.1 N KOH required toneutralize completely, the free fatty acids present in 1 g of fat oroil‖.
- Different fat samples may contain different amount of free fattyacids.
- On storage fats become rancid, which is caused by chemical or bacterial contamination? The amount of free fatty acids present is expressed as acid number or acid value. This acid number gives an indication about the age and quality of theoil.

Principle

- Hydrolysis of fats results in the liberation of free fatty acids from triglycerides. The amountof free fatty acids liberated is determined by titration against 0.1 NKOH.

Procedure

- Weigh a clean and dry 100 mL conical flask:(W_1).
- Pipette out 5 mL of oil into it and weigh again:(W_2).
- (W_2) - (W_1) gives the weight of the oiltaken.
- Add 20 mL of neutral alcohol into conicalflask.
- Heat the contents to boiling in a hot water bath (do not use an openflame).
- Titrate the hot solution against the 0.1 N KOH using phenolphthalein asindicator.
- The end point is the appearance of the pale pink color, which persists for half aminute.
- Record the volume of KOH consumed. Repeat the experiment for concordantvalue.

Calculation

- Weight of the oil taken, W=_____
- Volume of KOH consumed, V=_____
- Strength of KOH =0.1N
- Equivalent weight of KOH =56.1
- Acid Number = (V / W) x 0.1 x56.1

Result

- The acid number of the given sample of oil=_____

Titration

- **Titration of Oil + Alcohol vs 0.1N KOH**
- Indicator :Phenolphthalein

S.No.	Vol. of Oil taken (ml)	Burette Reading (ml)		Volume of KOH Consumed (ml)	Concordant Value (ml)
		Initial	Final		

Exercise No. - 7

Color and Precipitation Reaction of Proteins

The general properties of proteins are studied under two headings:

1.Colorreactions 2. Precipitationreaction

1. Color reactions

The color reactions are due to the reaction between one or more of the constituent radicals or groups of the complex protein molecule and the chemical reagent. All the proteins do not contain thesameaminoacidandsothevariouscolortestswillyieldreactionsvaryinginintensityofcolor according to the nature and amount of groups contained in the particular protein under examination.

BIURET TEST

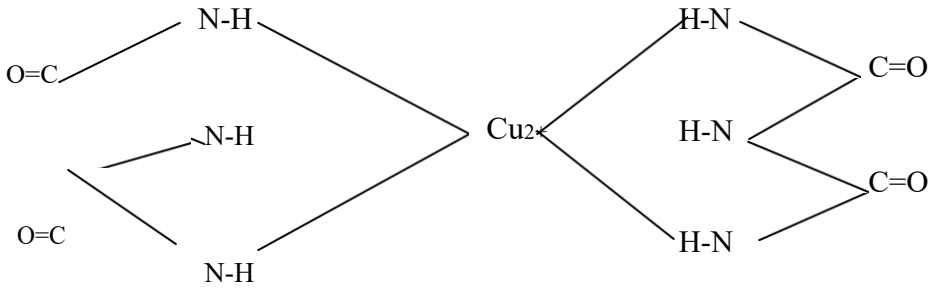
Principle

The test is positive for all the compounds containing more than one peptide linkage e.g proteins and their hydrolytic products (Meta proteins, peptones, polypeptides except dipeptides and amino acids).

Biuretreagent=5gmNaOHin100mldistilledwater+1%$CuSO_4$Solution(1gm$CuSO_4$dissolved in 100 ml distilledwater)

Method

Take 2 ml of protein solution and equal volume of 10% NaOH, mix thoroughly and then add 2 drops of 0.5% $CuSO_4$ solution drop by drop. A purple color due to the formation of biuret complex indicates the presence of peptide linkage.



Precautions

- Care must be taken that not more than 2 drops of $CuSO_4$ be added otherwise blue color will be developed instead of purple color. Peptones give pink color.

NINHYDINE TEST

This test is for the identification of α – amino acids, proteins and peptides**.**

Principle

- Ninhydrin, an oxidsing agent causes oxidative decarboxylation of α-aminoacids.
- It removes CO_2 and NH_3 and the product is one carbon lesser than the parent aminoacid.
- The reduced ninhydrin (hydrindantin) reacts with released NH_3 and one more molecule of
- Ninhydrin to produce a blue colored complex known as **"Ruhemann'spurple".**

Procedure

- To 1 ml of test solution add 2-5 drops of Ninhydrinsolution, mix well and heatit.
- Observe the development of pink or purple or violet bluecolor.
- Imino acids (proline and hydoxyproline) give a yellow coloredcomplex.
- This test also answers for proteins / peptides as they contain α-aminoacids.
- Amino acids will give negative results.

Observation

Experiment	Observation	Inference

Some other Color Reactions of protein:

1. Millon's Test- This test is specific for hydroxyl phenyl group or tyrosine

Principle-themercuricionsprecipitatetheproteinandformacomplexwithproteincontaining tyrosine. This complex turns red with nitrous oxide mercury is present in the reagent which precipitates the protein .The sodium nitrate then cause nitration of tyrosine. This compound isred coloredcomplex.

Reagent required:-

1. Millon's reagent- 150gm mercuric sulphate dissolved in 500ml distilled water then add168ml Conc. Sulfuric acid slowly by stirring and make final volume upto 1liter by distilledwater.
2. 1% solution of sodium nitrate. (1gm sodium nitrate dissolved in 100 ml distilled water)

Procedure-Take 3ml of protein solution in a dry test tube and add small quantity of millon's reagent. Boil it for1 minute, cool it and add 1% solution of sodium nitrate, warm gently. Appearance of red color solution or ppt indicates positive test.

Inference- the test is given by proteins containing tyrosine.

2. Xanthoproteic Test

Principle-The benzene ring in the amino acid is nitrated by heating with concentrate nitric acid or HNO3 and form yellow nitro compound. They turn orange with an alkali.

Reagent required

1. ConcentratedHNO3 2. Ammoniasolution

Procedure- Take 3ml of protein solution in a dry test tube then adds 1.5ml conc. Nitric acid, boil the solution and observe its color. Generally a yellow color forms then cool it and add small quantity of strong ammonia solution. The color of solution turns deep orange on an addition of alkali.

Inference- It indicates a positive test for an amino acid containing a benzene ring in the given protein. Amino acid tyrosine, tryptophan & phenyl alanine contains a benzene ring, so this test is given by them.

3. Hopkins cole Test- Proteins containing tryptophan gives positive test. This test is specific for indole group containing amino acid tryptophan. This test is also used to distinguish between gelatin and peptone. Gelatin is deficient in tryptophan amino acid so gives a negative test while peptones give positive test.

Principle- in the presence of concentrate H_2SO_4 indole group condenses with formaldehyde and gives a violet color complex compound

Reagent required-

1. Millon‘sreagent
2. Diluteformalin
3. Concentrated Sulfuric acid

Procedure-Take 3ml of protein solution in a test tube and add 3 drops of millon‘s reagent. Mix it and add 2-3 drops of dilute formalin then gently add 2ml of concentrate Sulfuric acid from side wall to test tube. Rotate the test tube between 2 palms a violet color ring at the junction of two liquids indicate positive test.

Inference- Positive test indicate the presence of peptones and protein containing tryptophan amino acid.

2. Precipitation reactions of Protein

Principle

Proteins can be precipitated from the solution by different reagents because of high molecular weight and are being colloidal in nature. These reagents are salts of heavy metals like $AgNO_3$, $ZnSO_4$ and $HgCl_2$, alkaloid reagents viz. picric acid, phosphotungstic acid, tannic acid, neutral salts like concentrated solution of ammonium sulphate, sodium sulphate and convert the proteins into suspensoids, which flocculate upon the addition of a few drops of salt solution, alcohols bring protein solution into iso-electric point, at which it is precipitated.

Proteins are precipitated from their solution by a variety of reagents.

(a). Precipitation byacids (b). Heatcoagulationtest (c). Precipitation bysalt

(a). Precipitation by Acids (Acetic acid Test) Principle- protein

are precipitated by acids by causing change in PH **Reagent -**1% acetic acid

(1ml acetic acid dissolved in 100 ml distilledwater)

Procedure-take 3ml of protein solution in a test tube and add drop by drop 1% acetic acid solution, appearance of thick white ppt indicates positive test.

Inference-Casein is present in the solution. It is confirmative test for Casein.

(b). Heat coagulationtest

Principle-When the solutions of protein are heated at their isoelectric pH, they get denatured. This process is known as Coagulation of protein by heat. Albumin and globulin are heat coagulable proteins where as gelatin and peptones are non- heat coagulable proteins.

Procedure- Take protein solution about 2/3 of test tube and heat the upper protein of solution by rotating the test tube, A turbidity in the upper portion indicate the positive result and compare with the lower unheated portion.

Inference- Positive heat coagulation test indicate the presence of albumin and globulin.

(c). Precipitation bySalt

1. **Half Saturation test with ammonium sulphate:** This test is used to distinguish between albumin andglobulin

Principle-proteins are colloidal in nature concentrated salt solution cause precipitation of proteins by dehydration.

Reagent- Ammonium sulphate

Procedure-Take 3ml of distilled water in a test tube and saturate it with ammonium sulphate separate the supernatant in another test tube, and in this add 3ml protein solution. This will make

half saturation. Shake vigorously and allow to stand for some time. Appearance of gelatinous precipitate indicates positive test and clear solution indicates negative test. **Inference-** The half saturation test is positive for globulin, gelatin and casein.

2. **Full saturation test**

Reagent- Ammoniumsulphate

Procedure- Take 3-5ml protein solution in a test tube and saturate it with solid ammonium sulphate. Keep the test tube for some time. Appearance of precipitate is indicative ofpositive test.

Inference-Albumin gives positive full saturation test.

Other Precipitation tests:

1. **Precipitation by salts of heavy metals**: Take 2 ml of dil. protein solution (egg white solution), add a few drops of 1 % $ZnSO_4$ drop by drop. A white precipitation is formed, indicating the presence of protein, which disappears on addition of excess of $FeCl_3$solution.
2. **Precipitation by alkaloid reagents**: Add 1 ml of Esbach's reagent (solution of picric acid andcitricacid)to1mlofproteinsolution.Ayellowprecipitationisformed,indicatingthepresence ofprotein.
3. **Precipitation by alcohol**

a) To 1 ml of egg white solution, add 2 to 3 ml of alcohol. White opalescent forms by dehydration, indicating the presence of protein. Filter after 10 min. Try to dissolve the residue by water, the precipitate does notdissolve.

b) Take 1 ml of protein solution in a test tube and 2 to 3 ml of alcohol in another test tube.Keep both the tubes in freezing mixture for 90 min and then mix the contents of two tubes. A white opalescent appears. Filter at once. Try to dissolve the residue on the filter paper and observe that the precipitatere-dissolves.

4. **Precipitation by acids:**

Protein precipitation by strong acids such as sulphuric acid, nitric acid, hydrochloric acid & also by weak acids such as acetic, tungestic acid etc. Protein can bind with acid ions & form insoluble salt such acid sulpho-protein, nitro-protein, protein-tungstate etc., which are insoluble.

Heller's Test: - To 2 ml of the protein solution, layer equal volume of Conc. HNO_3 along the inner sides of the tube. A white ring appears at the junction of the two phases due to precipitation of proteins. These meta-proteins of albumins and globulins are insoluble in strong mineral acid.

5. **Precipitation with strong alkali: -** Protein in solution are precipitation by strong alkali i.e. sodium hydroxide, ammonium hydroxide, potassium hydroxide etc resulting in the formation of white ppt.

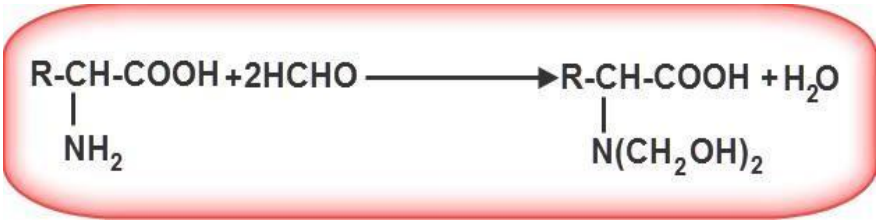
   Procedure: Take 1 ml of protein solution in a test tube, add equal amount of NaOH or KOH. White precipitate of protein in formed.

Exercise No. - 8

Estimation of Amino Acids (Sorenson's Method)

Principle

- The formaldehyde reacts with the amino group (-NH_2) of an amino acid and thereby prevents the formation of zwitter ions with carboxylgroups.
- This permits the carboxyl groups (-COOH) to exert maximum acidity, which can be measuredby titration against a standard NaOH solution, using Phenolphthalein as an indicator.



Procedure

Part I

Titration of amino acid + HCHO vsNaOH

- Pipette out 5 ml of amino acid solution (glycine) into a 100 ml conicalflask.
- Add 5 ml of formaldehyde solution and 2-3 drops of phenolphthalein asindicator.
- Titrate against the standard 0.02N NaOH solution till a permanent pale pink colorappears.
- Note down the volume of NaOH utilized as(A).
- Repeat the experiment for concordantvalue.

Part II

Titration of HCHO vsNaOH - Blank Titration

- Pipette out 5 ml of the same formaldehyde solution into another 100 ml conicalflask.
- Add 5 ml of distilled water and 2-3 drops of phenolphthalein asindicator.
- Titrate against the standard 0.02N NaOH solution till a permanent pale pink colorappears.
- Note down the volume of NaOH utilized as(B).
- Repeat the experiment for concordantvalue.

Calculation

- Volume of NaOH consumed by amino acid V_1 = (A) –(B)
- Normality of NaOH N_1 = 0.02N
- Volume of amino acid taken V_2 = 5.0ml
- Normality of amino acid N_2 = ? (Use the formula$V_1N_1=V_2N_2$)

- The concentration of amino acid in the given solution = N_2 x Eq.Wt of the amino acid = N_2 X 75 (Eq.Wt ofGlycine)=_____g/L.

Result

- The concentration of Glycine in the givensolutionis_____g /L

Observation

- **TitrationI:**
- MAINTITRATION
- AMINO ACID + HCHO vs. 0.02 NNaOH
- INDICATOR:PHENOLPHTHALEIN

S.No.	Volume of Amino acid taken(mL)	Burette Reading (mL)		Volume of NaOH Consumed (mL)	Concordant Value "A" (mL)
		Initial	Final		

- **TitrationII:**
- BLANK TITRATION
- HCHO vs. 0.02 NNaOH
- INDICATOR:PHENOLPHTHALEIN

S.No.	Volume of formaldehyde taken(mL)	Burette Reading (mL)		Volume of NaOH Consumed (mL)	Concordant Value "B" (mL)
		Initial	Final		

Unit-III

Veterinary Analytical Biochemistry

Exercise No. 1

Detection of Pathological Constituents in Urine

Introduction

In any pathological condition, analysis of urine is a useful diagnostic aid.

Commonly analyzed pathological constituents (substances) of urine are:

- Protein(Albumin),
- Reducing sugars(Glucose),
- Ketone bodies(Acetone),
- Bile (Bile salts and Bilepigments),
- Blood.
- Myoglobin
- Sediments

Most of these substances are present in trace amounts in normal urine also.

Chemical Examination of Urine (Qualitative)

1. PROTEIN(ALBUMIN)

Normal urine contains a trace of albumin, which is too slight to be detected by the usual procedures.Albuminuriaistheconditioninwhichreadilydetectableamountsoftheserumproteins are found in the urine. The proteinuria is most commonly due to the presence of serum albumin, since albumin is the most abundant of these serum proteins, and has the smallest molecular size, thus permitting the greatest diffusion through damagedmembranes.

There are two distinct forms of albuminuria VIZ. **renal albuminuria and accidental albuminuria.** Sometimes the terms-true albuminuria and false albuminuria – are substituted for these.Intherenaltype,thekidneysexcretethealbumin.Thisindicatesamoreseriousconditionand at the same time is more frequently encountered than the accidental type. Associated with renal albuminuria are usually altered blood pressure or kidney structures. In the accidental form of albuminuria, the albumin is excreted by the kidneys, but arises from the blood lymph or some albumin containing exudates coming into contact with the urine at point below the kidneys. The kidney disturbances are diagnosed by searching for epithelial casts or excessive red cells and white cell in concentratedurine.

A mild albuminuria may occur during the febrile stage of infectious disease and violent exercise. Foreign proteins injected into the blood stream are excreted through urine, and lead to some excretion of blood proteins also.

Biochemical Tests:

Heat coagulation test:

Principle: On heating there is precipitation of protein because of denaturation of protein.

Procedure: Take a test tube and fill it three forth with urine sample. Hold the tube from bottom and boil the upper portion of the urine for a minute.

Observation: The appearance of cloudiness indicates the presence of protein (Albumin) or phosphates. Addition of few drops of glacial acetic acid dissolves the precipitate if it is due to the presence of phosphates.

(b) Heller's nitric acid test:

Principle: The proteins are precipitated by the acid due to denaturation.

Procedure: Take 3 ml of nitric acid in a test tube and add few drops of urine sample from the side walls of the test tube, taking care to avoid mixing of the two solutions.

Observation: The appearance of white ring formed at the junction of the acid and urine indicates positive reaction for the presence of albumin.

Interpretation:

Physiological or functional protein/urea:

- Excessive muscularexercise.
- Emotionalstress.
- Ingestion of excessive amount ofprotein.
- 1st few days oflife.

Pathological protein/urea:

Pre-renal causes: The protein originates from non – renal condition and not due to primary renal disease e. g. Haemoglobinouria. Myoglobinuria

Renal Causes:

- Increase permeable ofglomerulus.
- Entire reabsorption ofprotein.
- Normally present in Glomerularfiltrate.
- Blood or exudates from renalorigin.
- Polycystickidney.

Post- renal:

- Ureteritis (inflammation ofurater).
- Cystitis
- Urethritis

- Urolithiasis (Stone inbladder)

2. GLUCOSE

Traces of this sugar may occur in normal urine but the amount is ordinarily too smallto bedetectablebythecommonqualitativetests.Thepresenceofreadilydetectableamountsofglucose in urine is known as **glucosuria.** The term glycosuria is frequently used but the expression more correctly refers to the presence of any sugar, not necessarily glucose in theurine.

Transitory glycosuria may be caused by a strong emotion such as fear, anxiety or excitement,whichstimulatestheadrenalsympatheticmechanismandraisesthebloodsugarbeyond the threshold‘ point. This condition occurs commonly in domestic animals owing to hyper excitability derived from restraints, strange surroundings etc. and these findings should persistonly as long as the animal continues to be in emotionalstate.

Small quantities of glucose may be found in nephritis because of impaired tubular reabsorption. **Renal glycosuria,** a benign condition caused by a lowered renal threshold for sugar, sometimes produces transitory or persistent traces of sugar. In diabetes mellitus, there is hyperglycemia, polyuria and glycosuria with normal renal threshold for glucose is noted.

Biochemical Tests:

If albumin is present remove it by heat coagulation and filtration before testing.

Test for reducing sugars (Benedict’s Test):

Principle: Thereducingsugar(glucose)formenediolinpresenceofalkaliwhichreducesthecupric ion of copper sulphate to cuprous ions that form the colorcompound.

Procedure: Take 5 ml of Benedict‘s reagent in a test tube and boil it for few minutes. Then mixes few drops of urine and boil it.

Observation: Formation of green, yellow, orange or red color precipitates indicates the presence of reducing sugar.

Interpretation:

Normal urine does not contain glucose. Although glucose passes through glomerulus by the kidney tubules.

Glycosuria with hyperglycemia:

- Glycosuria occurs in dog when blood glucose exceeds 180 mg/100 ofblood.
- Diabetesmellitus
- Acute pancreaticnecrosis
- Hyperadrenocortism

- Increase secretion or injection of adrenalhormones
- Excessive intake ofcarbohydrate
- Enterotoxemia inSheep
- Hyperthyroidism

Glycosuria without hyperglycema:

- Diabetesincipidus
- Antibiotics
- Lactose,pentose
- Ascorbicacid
- Chloralhydrate

3. KETONE/ACETONEBODIES

The acetone or ketone bodies include the compounds aceto-acetic acid, beta-hydroxy butyric acid and acetone. Aceto-acetic acid and beta-hydroxy butyric acid are primary products,the latter probably being formed by. Reduction of the former. Acetone, however, is a decaboxylated product of keto-acetic acid.

Aceto-acetic acid and beta-hydroxy butyric acid are either intermediate products in the breakdown of fatty acid chains or secondary combinations of 2 carbon fragments formed in this breakdown or closely related to it. Under normal conditions, the fatty acids in the animal body are oxidized completely to carbon dioxide and water and intermediate products do not appear to any great extent in the blood and urine. In certain abnormal conditions. However, the ketone bodies accumulateintheblood(ketonemia)andareexcretedintheurine(ketonuria)Thisgeneralcondition is known as**ketosis.**

Ketosis is apparently always associated with some abnormality of carbohydrate metabolism. Ketosis occurs in fasting or during carbohydrate deprivation and disappears when carbohydrate is fed. Pathologically, it is most severe in diabetes mellitus, when accumulation of ketonebodiesislargelyresponsibleforthedevelopmentofdiabeticacidosis.Ketosisalsooccursin fevers, pregnancy, ether and chloroform anesthesia, malnutrition, prolonged feeding of a carbohydrate poor diet, high in meat andfat.

Biochemical Test for ketone bodies (Rothera's Test):

Principle: Acetone, Acetoacetic acid and β- hydroxy butyric acid are collectively known as ketone bodies. The sodium nitroprusside decomposes into certain strong oxidizing agents in alkaline

solution. These oxidizing agents form purple color complex in the presence of diacetic acid and acetone.

Reagent: 100 gram Ammonium sulphate + 50 gram sodium carbonate + 3 gram sodium nitroprusside

Procedure: Take pinch of Rothera's powder in a test tube and add few drops of urine sample in it, and then add few drops of ammonia solution. Without mixing keep the tubes for few minutes.

Observation: Appearance of violet color indicates the presence of ketone bodies in urine.

Interpretation:

Ketosis: In pregnant or lactating cows. It is associated with hypoglycemia

Diabetes Mellitus. Associated with hyperglycemia.

Starvation or fasting.

Highly fat rich diet.

4. BILE

Both the pigments and the acids of the bile may be detected in the urine under certain pathologicalconditions.Aurinecontainingbilemaybeyellowishgreentobrownincolorandwhen shaken,foamsreadily.Thestainingofthevarioustissuesofthebodythroughthe absorptionofbile duetoocclusionofthebileductisprominentsymptomoftheconditionknownasicterusorjaundice. Bile is always present in the urine under such conditions unless the amount of bile reaching the tissues is extremelysmall.

Biochemical Tests:

Bile salts (Hay's Test): Bile salts are sodium and potassium salts of glycocholate and taurocholate.

Principle: Bile salt reduces the surface tension of urine and when sulphur powder sprinkled over urine surface, it gets sunk.

Procedure: Take5mlofurinesampleinatesttubeandsprinkleapinchofsulphurpowderoverthe surface ofurine.

Observation: If the sulphur powder immediately sinks through the urine, it indicates the presence of bile salts in urine sample.

(B) Test for Bile pigments (Gmelin's test):

Principle: Bile pigments are bilirubin and biliverdin. The test for bile pigments depends upon oxidation of bilirubin by acids to colored compounds of blue, green, violet, red and yellow.

Procedure: Take small quantity of urine and to this add 2-3 ml of conc. nitric acid from the side wall of the test tube.

Observation: Appearance of blue/green or violet ring at the junction indicates the presence of bile pigments.

Interpretation:

Always interpretate with association with specific gravity.

Causes:

Hepato-cellular diseases e.g. cirrhosis

Obstruction of bile duct.

5. BLOOD

Thepathologicalconditioninwhichbloodoccursintheurinemaybeclassifiedunderthe two divisions: **hematuria** and **hemoglobinuria.** In hematuria it is possible to detect not only the hemoglobinbutalsotheun-rupturedcorpuscles(intactRBC's)aswell,whereasinhemoglobinuria, the pigment alone is present. Hematuria is brought about through blood passing into the urine because of some lesion of the kidney or of the urinary tract below the kidney. Hemoglobinuria is broughtaboutthroughhemolysisthatisrupturingofthestromaoftheerythrocyteandtheliberation ofthehemoglobin.Thismayoccurinhemolyticjaundiceandotherprotozoandiseases.Itmayoccur astheresultofaburningaconsiderableareaofthebodyandthroughtheactionofcertainhemolytic poisons.

Test for blood in urine (Benzedine test): Pseudoperoxidase reaction

Principle: Hydrogen peroxide is decomposed by peroxidase activity of hemoglobin that liberates oxygen. This oxygen oxidizes benzedine to give a blue color solution. This color changes on exposure to air.

Procedure: Take 1 ml of solution of benzedine and add 2 ml of urine sample fallowed by 1 ml of 3% hydrogen peroxide.

Observation: Appearanceofgreenorbluecolorwithinfewminutesindicatesthepresenceofblood in the urine sample. This color quickly changes to brown within fewminutes.

Interpretation:

Blood in urine (Hematuria): Causes

- Acutenephritis
- Renalinfarction
- Passive congestion ofkidney
- Urolithiasis

- Ureteritis
- Urethritis
- Trauma tourethra
- Cystitis
- Protatitis

Severe infectious diseases:

- Leptospirosis – Canine
- Anthrax – Sheep, Goat,Cattle.
- Infection canine hepatitis-Canine.

Chemical agent:

Cu-poisoning, Hg-poisoning and kidney worms,

Hemoglobinuria: Hb in urine is due to excessive haemolysis of RBC‘s.

Diseases:

- Babesiosis
- ParturientHemoglobinuria
- BacillaryHemoglobinuria.

6. MYOGLOBIN

This heme pigment, derived from muscular tissue, is found in the urine after extensive destructionofmusculartissue,asfromcrushinginjuries.Urinecontainingmyoglobinresemblesthat containing blood; it may be smoky dark brown or red and will give a positive benzidine test. Red cells however, are notably absent, and the sediment may contain brown pigment cast. Myoglobin has a molecular weight only about one fourth that of hemoglobin which probably explains itsready diffusibility through the kidneymembranes.

Biochemical Test:

In case of much damage to the muscle tissue, myoglobin will be found in the urine. It differentiatesfromthehemoglobinbytheprecipitationtechnique.5mlofurineisaddedto2.8grams of ammonium sulphate and shaken well to dissolve. Then filter or centrifuge. If abnormal color is still present in the filtrate it indicates that myohaemoglobin is present because the hemoglobin is completely precipitated at 80 percent saturation with ammonium sulphate. To show that the remaining pigment is protein, it is precipitated with salicylsulphonic acid (1 ml urine +8 ml salicylsulphonic acid). Fresh urine sample is preserved by adjusting the pH 7.0 to 7.5with

diluteNaOH and is used for test.

7. SEDIMENTS

Theelementaryconstituentsoftheurinemaybedividedintotwoclassesviz.organizedand un-organized. The sediment is collected by centrifuging the urine at low speed or by allowing it to stand for some time in a conicalflask.

Un-organized sediments:

The more common sediments under this category consist of ammonium magnesium phosphate (triple phosphate), calcium oxalate, calcium sulfate, and sodium and ammonium urates. Lesscommonlyobservedarecalciumcarbonate,magnesium,phosphate,cysteine,leucine,tyrosine, hippuric acid, bilirubin, xanthine andmelanin.

Phosphates are often seen in conditions in which there is retention of urine particularly if there is infection of urinary tract. Calcium hydrogen phosphate is found in alkaline urine. It IS also found in acidic urine with pH ranging from 6 to 7.

Uric acid is quite common in acid urine. Crystals dissolve in NaOH but not in acetic acid or HCL. It is commonly found after sweating during exercise and in fever. Urate crystals are found usually pigmented. Cholesterol is found rarely. It is often seen in kidney and renal tract infections.

Biochemical Tests:

Centrifugeabout15mloftheoriginalunfilteredurine.Discardthesupernatantfluid.Shake up sediment with few drops of the fluid remaining in the tube. Transfer a drop of this to a slide and coverwithacoverslip.Examineunderthelowpowerandhighpowerofmicroscopeforthepresence of organized and unorganizedsediments.

Exercise No. 2

Assay of ALT(Alanine Transaminase) and AST(Aspartate Transaminase) in Serum

A. ESTIMATION OF ALT OR SGPT (Enzymatic method – Kinetic mode)

Principle:

- Kinetic determination of SGPT is done as per the followingreactions:

SGPT (ALT)

(1) α -Ketoglutarate + L-Alanine → L-Glutamate +Pyruvate

LDH

(2) Pyruvate + NADH + H^+ → L-Lactate +NAD^+

- The utilization of NADH is monitored at 340 nm, whose rate of disappearance is proportionalto the activity of SGPT. The rate of decrease in absorbance is measured at 340nm.

Specimen: Serum / plasma

Reagents:

- Reagent 1 (Tris buffer pH 7.8, L-alanine,LDH,)
- Reagent 2 (α –Ketoglutarate,NADH)

Preparation of working reagent:

Mix four Volumes of Reagent 1 with one volume of Reagent 2. This working reagent is stable for 30 days at 2 – 8 ^{0} C.

Procedure:

- Mark one clean test tube as(T):

S.N.	Reagents	T
1.	Working Reagent	1000 m l
2.	Serum	100 m l
3.	Distilled water	2000 m l

- Mix well and incubate for 1 minute at 37^{o}C. Read the change in absorbance per minute (Δ OD/ min.) during 3 minutes at 340 nm setting zero withD.W.

Calculation:

- SGPT activity (U/L) = (Δ OD / min.) x 1745 x3

Result:

- The SGPT activity of givenserum =_____U /L.

Clinical Significance:

- ALTisincreasedinhepatocellularinjuryindogandcat. Itisnotusefulinevaluatingchronicliver disease. It may be elevated in corticosteriod treatment. This enzyme is not useful in evaluating hepatic disease in horse, cow, sheep, goat andpig.

ESTIMATION OF AST OR SGOT (Enzymatic method – Kinetic mode)

Principle:

- Kinetic determination of AST (Aspartate amino transferase) is done as per thefollowing reactions:

$$\text{(1) } \alpha\text{-Ketoglutarate+L-Aspartate} \xrightarrow{\text{SGOT (AST)}} \text{L-Glutamate + Oxaloacetate}$$

$$\text{(2) Oxaloacetate + NADH + } H^{+} \xrightarrow{\text{MDH}} \text{L-Malate +} NAD^{+}$$

- The utilization of NADH is monitored at 340 nm, whose rate of disappearance is proportionalto the activity of SGOT. The rate of decrease in absorbance is measured at 340nm.

Specimen:

Serum / plasma

Reagents:

- Reagent 1 (Tris buffer pH 7.8, L-aspartate, LDH,MDH)
- Reagent 2 (α –Ketoglutarate,NADH)

Preparation of working reagent:

- Mix four Volumes of Reagent 1 with one volume of Reagent 2. This working reagent isstable for 30 days at 2 – 8 ^{0}C.
-

Procedure:

Mark one clean test tube as (T):

S. N.	Reagents	T
1.	Working Reagent	1000 ml
2.	Serum	100 m l
3.	Distilled Water	2000 m l

- Mix well and incubate for 1 minute at 37^{O}C. Read the change in absorbance per minute (Δ OD/ min.during 3 minutes at 340 nm setting zero withD.W..

Calculation:

- SGOT activity (U/L) = (Δ OD / min.) x 1745 x3

Result:

- The SGOT activity of givenserum =_____U /L.

Clinical Significance:

- Elevated levels of SGOT are found in myocardial infarction, in liver diseases, skeletalmuscle trauma and sometimes in renaldiseases.

Exercise No. 3

Assay of Acute Phase Proteins (A/G Ratio)

A. METHOD OF ESTIMATION OF PROTEIN

Principle:

Proteins in serum react with copper of Biuret Reagent in alkaline medium to form a blue purple complex with absorption maximum at 550 nm. **Sample:** Serum or plasma.

Reagents:

- Total Protein Reagent (Biuret Reagent – Potassium iodide, Potassium sodium tartarate, Copper sulphate,NaOH)
- Total Protein Standard(6g%)

Procedure:

Mark three test tubes as Blank, Standard and Test and proceed as follows:

S.No.	Reagents	Blank (B)	Standard (S)	Test (T)
1.	Biuret Reagent	1.0 ml	1.0 ml	1.0 ml
2.	Total Protein Standard	-	20 m l	-
3.	Serum	-	-	20 m l
4.	Distilled Water	2.0 ml	2.0 ml	2.0 ml

- Mix well and incubate at 37^0C for 10minutes.
- Measure the O.D of all the Standard and Test against Blank at 550 nm or using an yellowgreen filter.

Calculations:

$$\text{Total Proteins (g/dL)(X)} = \frac{\text{OD of(T)}}{\text{OD of(S)}} \times 6$$

$$\text{A/GRatio} = \frac{\text{(Albumin)}}{\text{(Total Protein) – (Albumin)}}$$

Method of Estimation: Biuret Method

METHODS OF ESTIMATION OF ALBUMIN: BCG DYE METHOD

Sample: Serum or Plasma

Principle:

- Albumin in serum binds with the dye Bromocresol Green (BCG) at pH 3.8 to form agreen colored complex, the absorbance of which is measured at 600nm.

Reagents:

- Albumin Reagent (BCG Reagent – Succinate buffer, Bromocresolgreen)
- Albumin standard (3g%)

Procedure:

Mark three test tubes as Blank, Standard and Test and proceed as follows:

S.No.	Reagents	Blank (B)	Standard (S)	Test (T)
1.	BCG Reagent	1.0 ml	1.0 ml	1.0 ml
2.	Albumin Standard	-	10 m l	-
3.	Serum	-	-	10 m l
4.	Distilled Water	2.0 ml	2.0 ml	2.0 ml

- Mix well and allow the tubes to stand at R.T for 1 min.
- Measure the O.D of all the Standard and Test against Blank at 600nm or using a redfilter.

Calculations:

$$\text{Albumin(g/dL)} = \frac{\text{OD of(T)}}{\text{OD of(S)}} \times 3$$

$$\text{A/GRatio} = \frac{\text{(Albumin)}}{\text{(Total protein) – (Albumin)}}$$

ABNORMAL PLASMA PROTEINS – HYPOPROTEINEMIA

Abnormal Protein Concentration

- The liver synthesizes of albumin, fibrinogen, prothrombin and most of the globulins particularly alpha and beta globulins.
- The gamma globulins are synthesized in the lymphoidorgans.
- The normal range of total protein levels in most of the animals ranges between **5 and 8g/dL.**
- Edema develops when the total protein concentration in plasma falls below5g/dL.

Hypoproteinemia: (decreased protein concentration)

- **Hypoalbuminemia with hypoglobulinemia:** It may be due to decreased concentrations of albumin, globulin orboth.
- Bloodloss
  - Due to proportional loss of all blood constituents, interstitial fluid moves into the circulatory system and dilutes the remaining blood causing a decrease in the level of albumin andglobulin.
- Protein losingentropy
  - During a variety of intestinal lesions both albumin and globulin leak from the intestinal wallinto the intestinal lumen and then are digested orexcreted.
- Severe exudative skindisease
  - This results from vascular permeability that allows both albumin and globulin to escape fromthe blood.
- Severeburns
  - These cause increased vascular permeability that can result in loss of both albumin andglobulin.
- Effusivedisease
  - This results in the accumulation of body cavity fluids with high protein concentrations thatcan result in decreased albumin and globulinconcentrations.
  - The decrease depends on the degree of increased vascular permeability.
  - Hypoalbuminemia with normal to increased globulin concentrations.
  - The deceased albumin concentration can result from either decreased production orincreased loss ofalbumin.
  - If the concentration of globulin is increased the total protein level may benormal.
- Decreased production of Albumin can occur in the followingdisorders:
  - Hepatic Failure
  - Starvation
  - GastrointestinalParasitism
  - Mal-absorption
  - Exocrine pancreatic insufficiency(EPI)
    - Inadequate digestion of dietary proteins can result from EPI, in which amino acids are not liberated from the protein by digestion in the intestine, so they are not available forabsorption.
    - Decreased albuminproduction.
- Increased loss of proteins can occur in the followingdisorder
  - GlomerularDiseases

- Albumin are smaller than globulin, they leak more readily through damagedglomerular membrane.

ABNORMAL PLASMA PROTEINS – HYPERPROTEINEMIA

Hyperproteinemia: (Increased Protein Concentration)

Hyperalbuminemia and Hyperglobulinemia

- *Causes*
  - Loss of water from the blood causes increased concentrations of albumin andglobulin.
  - The albumin: globulin ratio is not altered because both fractions are concentratedequally.

Hyperglobulinemia

- It depends on the type of globulin that is increased: Increased gamma globulinconcentration.
- Acute inflammation is the most commoncause.
- Concentrations of several proteins in the globulin fraction (e.g., Ceruloplasmin, haptoglobin, and alpha2 macroglobulin) are increased. These proteins are collectively called as acutephase proteins.
- Increased beta globulin concentrations can occur with acute inflammation, nephroticsyndrome, liver disease and immuneresponse.
- Concentrations of several acute phase proteins in this fraction (e.g., C-reactive proteins, complement, ferritin) increase during acuteinflammation.
- Increased gamma globulin concentration: This fraction includes most of theimmunoglobulins.
- Increasesingammaglobulinconcentrationaretermedasgammopathiesandtheyaredividedinto polyclonal(havebroadbasedpeakinthebetaandgammaregions)andmonoglonalgammopathies (haveanarrowbasedelectrophoreticpeakinthebetaandgammaregions),whichsuggestschronic inflammatory diseases (e.g., chronic bacterial, viral, fungal or rickttsialdisordrer, parasitism (cutaneous parasites), cancer and immune mediateddiseases.
- Multiple myeloma is due to the proliferation of single clone of Blymphocytes.
- This clone produces a homogenous type (monoclonal immunoglobulin) of protein called as paraprotein orM-component.

A/G ratio

- A/G ratio provides a systematic approach to the interpretation of proteinvalues.
- **Normal A/Gratio**
  - Dehydration with water loss results in hyperproteinemia without a change in the A/Gratio.
  - Albumin and globulin fractions are increasedproportionately.
  - Excess fluid intake or fluid therapy is a simple cause of hypoproteinemia. This is due tothe dilution.

- **Decreased A/Gratio**
  - It is generally due to decreased level of albumin and increased level ofglobulins.
  - The conditions resulting in the reduced level of albumin and increased level of globulinshave been discussedearlier.
- **Increased A/Gratio**
  - Generally albumin is not produced inexcess.
  - Any increase in the level of albumin is due to hemoconcentration as a resultof dehydration. Decreasedglobulins
- Newborn animals are physiologically hypoglobulinemic (failure of passive transfer ofcholostral antibodies).
- When there is a failure in the formation of gamma globulins (Immunosuppression or immunodeficiency)

  The other causes are blood loss and protein losing enteropathy

Exercise No. 4

Estimation of Total Serum Cholesterol

Introduction

Cholesterol is the most abundantly occurring sterol in the animal kingdom from whichall other steroid components are derived. The chemical structure is shown below and is chemically classified as Cyclopentanoperhydrophenanthrene nucleus containingsteroid.

Cholesterol in blood is present in two forms, free cholesterol, as well as esterified cholesterol. In the plasma both free and esterified cholesterol are found while in the red cellsonly thefreeformappears.Plasmaispreferredtowholebloodforanalysis,sincepathologicalvariations in the amount and in the distribution between free and ester forms occur largely in the plasma fraction.

In the presence of acetic anhydride and conc. H_2SO_4, sterols in general develop colors rangingfromblueto green.Althoughtheexactnatureofchromophoregroupsarenotknown,itis knownthatundertheanhydrousconditionsanumberofmolecularrearrangementstakeplace.This iscalled***Liebermann–Burchard***testandisusedforthequantitativeestimationofcholesterol.
Different methods of cholesterol estimation are given below:

LIEBERMANN – BURCHARD METHOD

Estimation of blood or serum cholesterol is generally employed by this method. This is a highly sensitive, rapid and precise method.

Objective: Estimation of blood/serum cholesterol using the Liebermann – Burchard reaction.

Principle:

Aceticanhydridereactswithcholesterolinachloroformsolutiontoproduceablue–green color. The reaction probably includes esterification of the hydroxyl group in the third position as well as other rearrangements in themolecule.

Bloodorserumisextractedwithanalcohol–acetonemixturewhichremovescholesterolandother lipidsandprecipitatesprotein.Organicsolventisremovedbyevaporation,dryresidueisdissolved in chloroform and then cholesterol isestimated.

Dehydrated cholesterol + acetic anhydride → Blue green colored compound

Materials:

1. Alcohol – acetone mixture(1:1)
2. Chloroform
3. Acetic anhydride – sulfuric acid mixture (30:1, mix just beforeuse)
4. Cholesterol standard (0.4 mg/ml inchloroform)

5. Blood orserum
6. Test tubes, tube stand, water bath, spectrophotometer, centrifuge, centrifugetubes

Procedure:

1. Take 10 ml of alcohol – acetone solvent and add 0.2 ml of serum or blood in a centrifugetube.
2. Immerse the tube in a boiling water bath with shaking until solvent begins toboil.
3. Remove the tube, cool to room temperature andcentrifuge.
4. Decant the supernatant in a test tube and evaporate to dryness on a boiling waterbath.
5. Cool and dissolve the residue in 2.5 mlchloroform.
6. Take three test tubes and label them _T‘ for Test, _S‘ for Standard and _B‘ forBlank.
7. Take 2.5 ml cholesterol solution in T, 2.5 ml cholesterol standard in S and 2.5 ml chloroformin Btube.
8. Then mix 2.5 ml acetic anhydride-sulfuric acid mixture in eachtube.
9. Mix thoroughly; leave the tubes in dark at room temperature and read the O.D. of testand standard against the blank at 680 nm using redfilter.

Calculation:

$$\text{Concentration of cholesterol (Mg/dl)} = \frac{\text{O.D. of Test}}{\text{O.D. of Standard}} \times \text{Conc. of Standard.}$$

Result:

Concentration of cholesterol in the given blood / serumsampleis_____mg /dl.

HENRY METHOD

It is a modified method of LibermannBurchard method. Dehydrated cholesterol reacts with acetic acid to form a brown colored compound. **Objective:** Estimation of cholesterol in serum / plasma.

Principle:

Dehydrated cholesterol reacts with acetic acid to form a brown colored complex. Intensity of brown color depends upon the conc. of cholesterol in the sample.

Dehydrated cholesterol +Aceticacid complex (Cholesterol + H_2SO_4) → Browncolored

Materials:

1. Ferric chloride – glacial acetic acid reagent (0.05%)
2. Conc.H_2SO_4

3. Cholesterol standard (0.4 mg/ml in glacial acetic acid)
4. Blood serum orplasma.
5. Test tubes, test tube stand, water bath, spectrophotometer, centrifuge, centrifugetube.

Procedure:

1. Take 4.9 ml $FeCl_3$ – acetic acid reagent in a centrifuge tube and add 0.1 ml plasma / serum.
2. Mix well and keep at room temperature for 10min.
3. Incubate the tube in water bath at 60^oC for 2min.
4. Cool, mix and centrifuge for about 5 min. at 2000 rpm. Proteins are precipitated andcholesterol will remain in thesupernatant.
5. Prepare three test tubes as givenbelow:

Ingredients	B (Blank) ml	S (Std.) ml	T (Test) ml
Supernatant (Cholesterol)	-	-	2.55
$FeCl_3$ Acetic acid	2.55	2.5	-
Cholesterol std.	-	0.05	-
Conc. H_2SO_4	1.5	1.5	1.5

6. Mix the components and read O.D. of test and std. against blank at 560 nm using greenfilter.

Calculation:

$$\text{Concentration of cholesterol (mg/dl)} = \frac{\text{O.D. of Test}}{\text{O.D. of Standard.}} \times \text{Conc.ofStandard.}$$

Result:

Concentration of Cholesterol in the given plasma /serumis_____mg /dl.

Normal Values (mg/dl)

Cattle	Buff.	Sheep	Goat	Pig	Horse	Dog	Cat	Chicken	Human
35-160	40-100	40-60	65-135	115-120	40-170	110-250	70-175	183.8 (Av.)	150 – 220

Interpretation:

- **Hypercholesterolemia:** It is observed in nephrosis, diabetes, obstructive jaundice,myxoedema and hypothyroidism. In atherosclerosis the value may or may not beelevated.
- **Hypocholesterolemia:** It is observed in hyperthyroidism, anaemia and haemolytic jaundice. Similar findings may be seen in mal-absorption syndrome, severe wasting and acuteinfection.

Exercise No. 5

Estimation of Blood Urea Nitrogen (BUN)

Introduction

Urea is the detoxification product of the ammonia derived from the deamination of amino acids. Urea is therefore the most common nitrogen-containing end product of protein catabolism. Its synthesis from ammonia occurs in the liver (via the urea cycle), and it is then excreted by the kidneys. Urea is free to pass through all membranes of the body and is equally distributed in the body water. The concentration of urea inside red cells is slightly less than in plasma due to the presence of large amounts of hemoglobin inside the cells. Whole blood urea concentration is therefore slightly less than plasma (or serum) urea. Therefore, usually serum is used instead of whole blood for the determination of urea. The familiar term BUN (blood urea nitrogen) persists in spite of the fact that it is serum urea nitrogen which is usually measured.

Inmostclinicalsituations,changesinurealevelsaremoredependentuponkidneyfunction than upon liver function. Increased urea levels are associated with renal diseases as well as dehydration, diabetic coma, hypoadrenal crisis, gastrointestinal haemorrhage andcirculatory collapse. Decreased values are observed in some cases of severe liverdiseases.

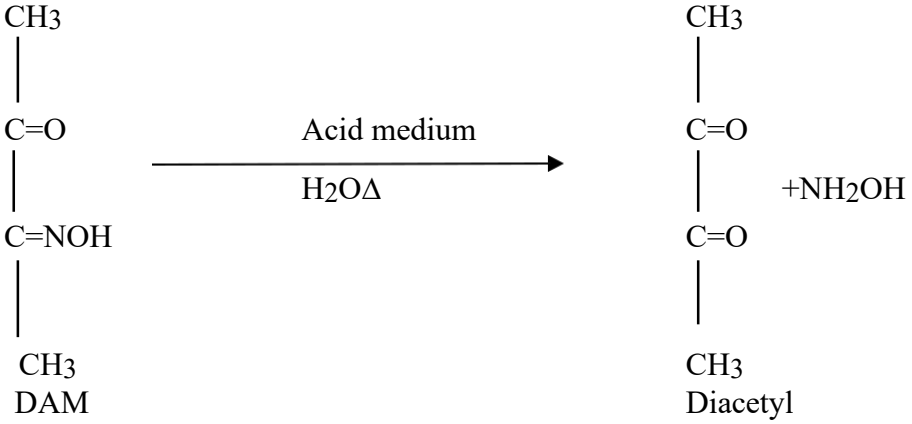
Urea is often estimated by DiacetylMonoxime (DAM) Method.

BLOOD UREA NITROGEN BY DAM METHOD

Objective: Quantitative determination of urea in plasma / serum (Diacetyl – Monoxime Method (DAM method).

Principle:

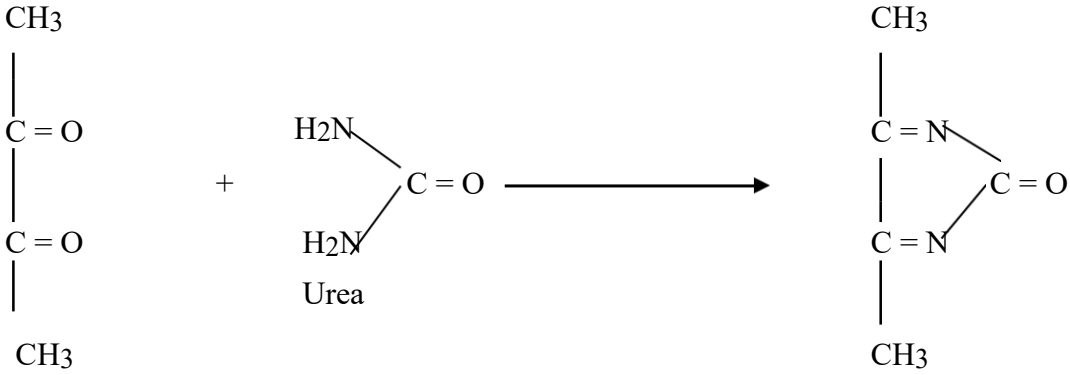
Urea reacts with diacetylmonoxime in acidic medium at 95^{o}C – 100^{o}C to give pink colored complex. Ferric ions are used to oxidize hydroxylamine formed in the reaction. Absorbance of pink end product is measured at 520$\pm$ 15 nm. The intensity of color developed is proportional to the conc. of urea present in the specimen.



DAM

Hydroxyl amine

Diacetyl



4, 5-Dimethyl-2-imidozolone (pink)

Materials:

- Diacetylmonoxime: 1.56 g diacetylmonoxime in 250 ml dist.water.
- Thiosemicarbazide : 41 mg thiosemicarbazide in 250 ml dist. water. Keep inbrown bottle.
- Ferric chloride reagent: 324 mg ferric chloride in 10 ml of 56% orthophosphoricacid. Keep in brownbottle.
- 20% Sulphuric acid(V/V).
- Acid reagent: To 1000 ml of 20% sulphuric acid, add 1 ml of ferric chloridereagent.
- 10% Trichloroaceticacid.
- Preservative diluent for standard: Dissolve 40 mg phenyl mercuric acetate in about 250 ml. distilled water with heating. Transfer the solution into a measuring cylinder. Add 0.3 ml of conc. sulphuric acid and make the volume to 1litre withwater.
- Standard urea: 10 mg urea in one litre of preservative diluents (1 ml = 0.01mg).
- Test tube, Test tube stand andSpectrophotometer.

Procedure:

1. Take 3.4 ml of dist. water in a centrifuge tube, add 0.1 ml blood. Mix. Add 1.5 ml of10% trichloroacetic acid and mix. Left for 5 minutes. Centrifuge it, collect thesupernatant.
2. Take three test tubes and label them _T‘ for Test, _S‘ for Standard and _B‘ forBlank.
3. Take 1.0ml blood supernatant in T, 1.0 ml urea standard in S and 1.0 ml D. water in Btube.
4. Then mix 1.0 ml Diacetylmonoxime reagent, 1.0 ml thiosemicarbazide and 3.0 ml acidreagent in eachtube.
5. Mix and place all the tubes in boiling water bath for 15 minutes and cool the tubes inwater.
6. Read the O. D. of standard & test against blank at 540 nm using greenfilter.

Calculation:

$$\text{Urea (mg / 100 ml of blood)} = \frac{\text{O.D of Unknown}}{\text{O. D. of Standard}} \times 0.01 \times \frac{100}{0.02}$$

Result: The bloodcontainsurea_____mg /dl.

Normal values (mg/dl):

Cattle	Sheep	Goat	Pig	Horse & Dog	Cat	Human	Chicken
12-56	17-42	27-60	17-52	22-42	40-60	16-50	0.8-2.14

Interpretation:

- Blood urea is lower in pregnant than in normal non-pregnant individuals. Low level of urea can be observed in nephrosis, some case of liver diseases and deficiency of protein indiet.
- High blood urea levels are observed in all forms of kidney disorders, severe vomiting,diarrhea, intestinal obstruction, colitis, diabetes, haematemesis, and coronarythrombosis.

Blood Urea Nitrogen (BUN)

- The BUN test measures the amount of nitrogen contained in theurea
- Urea is a relatively nontoxic substance formed in the liver as a means of disposing ofammonia from proteinmetabolism.
- Urea is filtered by theglomerulus.
- The real concentration of urea is BUN x2.14.
- Normal blood urea nitrogen is 8-25 mg/dL (2.9-8.9mmol/L).
- Blood urea levels are sensitive indicators of renal disease, becoming elevated whenrenal function drops to around 25-50% ofnormal.
- By definition increased BUN isAzotemia.
- It is due either to increased protein catabolism or impaired kidneyfunction.
  - Increased protein catabolism resultsfrom:
    - A really big protein meal (Kansas City steak,etc.)
    - Severe stress (myocardial infarction, high fever,etc.)
    - Upper GI bleeding (blood being digested andabsorbed)
  - Impaired kidney function may be "prerenal", "renal", or"postrenal".
    - Prerenal azotemia results from underperfusion of the kidney: dehydration, hemorrhage, shock, congestive heart failure; glomerulonephritis is likely also to be "prerenal" if mild, since it compromises renal blood flow more than tubularfunction.
    - Renal azotemia has several familiar causes: acute tubular necrosis, chronicinterstitial nephritis, some glomerulonephritis,etc.
    - Postrenal azotemia results from obstruction of urinary flow: prostate trouble, stones,surgical mishaps, tumors.
- In acute renal failure, BUN increases around 20 mg/dL each day (*estimates vary; rangeof increase is 10-50 mg/dLdaily).

Exercise No. 6

Estimation of Serum Creatinine

Creatine and Creatinine are metabolized in the kidneys, muscle, liver and pancreas. Structurally, Creatine is a methyl guanidine acetic acid which makes up to 98% of total muscle mass and plays a crucial role in muscle contraction. Creatine is excreted in the form ofanhydride, i.e. Creatinine. Serum Creatine concentration is increased in muscular atrophy and following extremity amputation. Excessive proteinuria results from impaired tubular Creatine reabsorption. Determination of Creatinuria has a diagnostic value only in case of atrophy and in muscle regenerationinmyopathies.Creatinephosphateundergoesspontaneousbreakdowninmusclecells to formCreatinine.

The loss of water molecule from Creatine results in the formation of Creatinine. It is transferred to the kidneys by blood plasma and it is eliminated from the body by glomerular filtration and partial tubular excretion. Serum concentration of Creatinine primarily depends on glomerular filtration. Plasma and urine Creatinine level is a fairly constant value which is related to total muscle mass. As Creatinine is endogenously formed and is not reabsorbed in the tubules, serum Creatinine is a reliable indicator of glomerular function.

Creatinine

- This breakdown product of creatine phosphate is released from skeletal muscle at a steady rate.(Only a small amount comes from meat in thediet).
- It is filtered by the glomerulus, and a small amount is also secreted into the glomerular filtrate by the proximal tubule. (hence at low GFR's, the usual reciprocal relationship breaks down and creatinine tends to underestimate how low the GFR hasgotten).
- Creatinine is generally considered a somewhat more sensitive and specific test of renal function thanBUN.
- Normal serum creatinine is 0.6-1.5 mg/dL (53-133micromoles/L).

METHOD OF ESTIMATION - ALKALINE PICRATE METHOD

- Alkaline picrate method is used to estimate the creatinine concentration in serum. But itmeasures some non-creatininechromogens present in plasma. It can be rectified byusing Lloyd‘s reagent, which measures only the true creatinine.

Principle

- Creatinine reacts with the picric acid to produce a colored compound, creatinine alkaline picrate. The change in absorbance is proportional to creatinineconcentration.

Reagents composition

- Creatinine dyereagent
  - Picric acid - 8.73mmol/L
  - Surfactant
- Creatinine base reagent
  - Sodium hydroxide - 300mmol/L
  - Sodium phosphate - 25mmol/Lab

Laboratory procedure

	Standard	**Sample**
Working reagent	1000 µL	1000 µL
Standard	100 µL	-
Sample	-	100 µL
Mix and read the optical density (T_1) 6 seconds after the sample or standard addition. Exactly after 6 seconds after the first reading take second reading (T_2).		

Calculation:

Creatinine conc. (mg/dL) = ($T_2 – T_1$) of sample) / ($T_2 – T_1$) of standard) X 2

Result:

Interpretation:

- TheincreasedlevelofCreatinineindicatesfunctionalimpairmentofkidney.TherateofCreatinine excretionisinfluencedbyGFRandanyotherabnormalitythatdecreaseGFRresultinanincrease in concentration ofCreatinine.
- In the earlier stages, Creatinine clearance test is a sensitive index of impaired renalfunction.
- The prerenal factors that increase blood urea have less influence on Creatinine concentration. Hence for diagnosis of renal diseases, serum Creatinine is preferred over urea estimation. In addition to renal disease, elevated level of serum Creatinine and Creatinuria may be observed in extensive muscledestruction.

Exercise No. 7

Estimation of Serum Bilirubin (Direct, Indirect & Total)

Introduction

Bilirubin(abilepigment)isanormalmetaboliteproducedbydegradationofhemoglobin, the major protein of red blood cells. Bilirubin is transported to liver through blood where it is conjugated to glucuronic acid. Conjugated bilirubin is soluble in aqueous system and ismeasured as direct bilirubin. Unconjugated bilirubin is insoluble in aqueous system and is solubilized with methanol.

If erythrocytes are destroyed at abnormally high rate or liver fails to conjugate bilirubin, the yellow pigment may accumulate in the blood giving a yellow color to the skin. Hyperbilirubinemia may occur in diseases of the liver or bilary tract, and also in extrahepatic conditions of hemolytic nature, such as those accompanying infectious diseases, pernicious anemia, hemolytic anemia, hemorrhage etc. Low values for bilirubin may be found in secondary anemia.Thedeterminationofbilirubinmaythereforebeofimportanceinthediagnosisofjaundice or differential diagnosis ofanemia‘s.

Serum bilirubin can be quantitatively estimated by Malloy and Evelyn method.

MALLOY AND EVELYN METHOD

Objective: To estimate serum bilirubin using Malloy and Evelyn method.

Principle:

Conjugatedbilirubin(Direct)coupleswithdiazotizedsulfanilicacid,toformazobilirubin, a red purple product in acidic medium. Unconjugated bilirubin (indirect) is diazotized only in the presence of its solvent. The red purple colored azobilirubin produced in the presence of methanol originates from both direct and indirect fractions. Thus it is the total bilirubin concentration. The difference of total and direct bilirubin gives indirect or unconjugatedbilirubin.

The intensity of red purple color is measured colorimetrically and is proportional to the conc. of the fraction of bilirubin.

$$\text{Bilirubin} + \text{Diazotized sulfanilic acid} \rightarrow \underset{\text{(Redpurple)}}{\text{Azobilirubin}}$$

Materials:

1. Bilirubin reagent (Absolutemethanol)
2. Diazo blank Hydrochloric acid, 1.5% v/v withwater.

3. Diazoreagent

   Solution A: Dissolve 1 g of sulphanilic acid in 15 ml of conc. HCl and make the volume to 1 litre with distilled water.

   Solution B: Dissolve 0.5 g of sodium nitrite in water and dilute to 100 ml. Prepare fresh after a month.

   Just before use, the diazo reagent is prepared by mixing 0.3 ml of solution A and 10 ml of solution B.

4. Stock standard bilirubin solution: Dissolve 10 mg of bilirubin in chloroform and dilute to 100 ml with chloroform. If require slightly warm the solution to dissolve thebilirubin.
5. Working standard bilirubin solution: 10 ml of stock standard bilirubin solution is diluted to100 ml with chloroform. The solution contains 0.02 mg of bilirubin per 2ml.
6. Plasma /serum.
7. Test tubes, test tube stand, pipette, andspectrophotometer.

Procedure:

1. Take four test tubes labeled TT, TB, DB, DT and mix the solution as given in the tablegiven.

Ingredients	**TB (Total Blank) ml**	**TT (Total Bilirubin) ml**	**DB (Direct Blank) ml**	**DT (Direct Test) ml**	**Standard**
Serum	0.10	0.10	0.10	0.10	0.1
Distilled H_2O	1.40	1.40	2.65	2.65	1.40
Diazo blank	0.25	-	0.25	-	-
Diazo reagent	-	0.25	-	0.25	0.25
Bilirubin reagent (methanol)	1.25	1.25	-	-	1.25

2. Mix well and read O.D. of DT and DB after 1.0 min at 540 nm against distilledwater.
3. Keep TB and TT for about 30 min. and read O.D. at 540 nm against distilledwater.
4. Read the O.D. of standard at 540 nm using greenfilter.

5. Calculation:

Bilirubin conc. (mg/ dl) =mg%

$$\text{TotalBilirubin(A)} = \frac{\text{O.D. of TT} - \text{OD of TB}}{\text{O.D. of Standard}} \times \text{Conc. ofStandard}$$

$$\text{Direct Bilirubin(B)} = \frac{\text{O.D. of DT} - \text{OD of DB}}{\text{O.D. of Standard}} \times \text{Conc. ofStandard}$$

Indirect bilirubin = (A) – (B)

Result: In the given sample,

- TotalBilirubinis---------- mg%
- Direct (Conjugated)Bilirubinis---------- mg%
- Indirect(Unconjugated)Bilirubinis:--------- mg%

Normal Bilirubin concentration in plasma:

S.N.	Species	Range (mg/dl)		
		Total	Direct	Indirect
1.	Cow	0.01 - 0.5	0.04 - 0.44	0.03
2.	Dog	0.10 - 0.5	0.06 - 0.12	0.01 - 0.49
3.	Cat	0.15 - 0.50	-	-
4.	Horse	1.0 - 2.0	0-0.4	0.2 - 2.0
5.	Goat	0-0.1	-	-
6.	Sheep	0.1 - 0.5	0 - 0.27	0 - 0.12
7.	Pig	0-10	0 - 5.13	0-0.3
8.	Human Being	0.2 - 1.2	1.0 - 0.4	0.2 - 0.7

Interpretation:

- Hyperbilirubinaemia is a characteristic ofjaundice.
- In hemolytic and neonatal jaundice, unconjugated bilirubin level increaseswithout corresponding increase in conjugatedbilirubin.
- In viral hepatitis, toxic hepatitis and cirrhosis, there is overall damage to liver cells andhence there is increase in both conjugated and unconjugated bilirubinlevels.
- In obstructive jaundice, there is an increase in conjugated bilirubin and a normal levelof unconjugatedbilirubin.

Exercise No. 8

Principle and Procedure of Various Diagnostic Tests

Clinical pathology

- Clinical Pathology is a "Subspecialty of pathology that deals with the use of laboratory methods (Clinical biochemistry, microbiology, haematologyetc) for the diagnosis and treatment of a disease or disease condition." In general terms, it is the study of disease in the clinical/bedside environment by use of laboratory assays. It is referred as service pathology and has become importantin
    - o Arriving at correctdiagnosis
    - o Following the course ofdisease
    - o Evaluating thetherapy
    - o Giving a dependable prognosis of the diseaseconditions
- According to a well-known diagnostician, ―A clinician who says that he does not need a clinical laboratory aid is ill informed/uninformed and a clinician who depends completely on clinical laboratory findings for diagnosis is inexperienced. In either case the patient is indanger.

DIAGNOSTIC METHODS

- Diagnosis (Dia-through; gnosis-knowledge) is an art of determination of the nature of diseasei.e. etiology, lesions, symptomsetc.

Classification

- Diagnosis can be classified into three types based on the method ofexamination.

o ***Clinical diagnosis:*** This is based on inspection of animal by observing the clinicalsymptoms.

o ***Physical diagnosis:*** This is based on the examination of animal by physical methods like palpation, percussion, auscultationetc.

o ***Laboratory diagnosis****:* This is based on the laboratory findings like examination ofclinical samples like blood, faeces, urineetc.

TYPES OF CLINICAL LABORATORY

- Basic clinical laboratory
- Complete clinicallaboratory

Basic clinical laboratory

- This is an adjunct to any hospital for routine screening of specimens collected by the clinician. Examination of faecal samples, urine, skin scrapings, nasal washing and screening of wetfilms

are usually done as a routine in all laboratories.

Complete clinical laboratory

- It is a full-fledged laboratory having the followingsections.
  - ***Clinical pathology section****:* Deals with examination of body fluids (blood, urine,cerebrospinal fluid, ascitic fluid etc) and also report on biopsyspecimens.
  - ***Clinical microbiology section:*** Deals with entire range of immunological, cultural andantibiotic sensitivitytests.
  - ***Clinical parasitology section:*** Deals with identification of the parasites, parasitic eggs/oocystsof **clinical importance (Both ecto and endoparasites).**
  - ***Clinical Biochemistry section:*** Deals with various biochemical tests like blood urea nitrogen (BUN), creatinine etc. Alterations in biochemical parameters in disease condition arecalled ―Biochemical lesions‖ and they form the basis of diagnosis of many important ailments. (Examples – Van den Bergh test in jaundice, blood sugar in diabetes mellitus, Aspartate aminotransferase (AST) in myocardial infarction, BUN and creatinine in kidney disorders, Creatinine phosphokinase (CPK) in muscle damage, Alanine aminotransferase (ALT) in liver dysfunction).
- Immunohaematology or blood group studies are gaining importance in veterinaryfield.

ERRORS AND POINTS TO BE REMEMBERED IN LABORATORY

Points to be remembered with regard to clinical laboratory examination

- All specimens should be properly collected andlabelled.
- A history sheet giving details of the case should always accompany every specimen.*viz*. Owner‘s name,address,detailsofanimal(kindofanimal,breed,color,age,sex)history,tentativediagnosis, name of the clinician referred and type of samplesent.
- Sample should be as fresh as possible and preserved in a correctmanner.
- Repeat samples should be examined wherever possible. It is always better to base the interpretation of data built on 3 or 4 screenings(examination).
- Tests,whicharesimpleandeasilyperformedinaminimumtime,shouldbeselected.Complicated tests are resorted only when they are absolutely essential for establishing adiagnosis.
- Promptness in sending the results of tests is of paramount importance for early diagnosisand treatment.
- Specimens register should be properly and neatly maintained giving all the relevant particularsof thecase.

Errors in clinical laboratory

- Errors in samplecollection
- Laboratory bencherror
- Errors in reporting or clericalerrors.

All errors should be carefully avoided to enhance the reliability of the laboratory results. _Reliability‘ is a comprehensive term to include accuracy, sensitivity and reproducibility.Inorder toenhancereliability,comprehensivequalitycontrolprogrammeshouldbeintroduced.Periodical cross checking with a standard laboratory, test checking with a different staff and introduction of dummy samples in a batch are some of the useful methods that may help to maintain the quality ofwork.

Exercise No. 9

Renal Function Tests

The kidney functionsincludes:

- Removal of metabolic waste products from thebloodstream.
- Regulating the body‘s water balance, and maintaining the pH (acidity/alkalinity) ofthe body‘s fluids.
- Kidney produces calcitrol required for calciummetabolism.
- It produces erythpoitein for RBCproduction.
- Excretes Phosphorus and potassiumions.

A number of clinical laboratory tests that measure the levels of substances normally regulated by the kidneys can help to determine the cause and extent of kidney dysfunction. Urine and blood samples are used for these tests.

ASSESSING RENAL FUNCTIONS

The kidney function tests include:

- Blood test - Serum creatinine analysis, Urea estimation,Hemoglobin, Phosphorus, potassium and calciumestimation.
- Urinalysis
- Clearancetest
- Renalpyelography
- Ultra soundscan

Investigations

Urinalysis

Appearance	Blood, colour, turbidity.
Specific gravity	Sticks measure ionic particles only, not glucose.
pH	Normally acidic, except after a meal.
Glucose	Its presence may indicate increased blood glucose, or tubular disorder
Protein	Its presence may be due to: glomerular leak, raised serum proteins, Bence-Jones proteins myoglobulin, or of -renal origin
Microscopy	UTI will show polymorphs with no casts acute glomerulonephritis will show lls and cells & casts Chronic glomerulonephritis shows little sediment

Glomerular Filtration Rate (GFR)

- This is the most frequent test of renalfunction.
- GFR varies as a function of normal physiology as well asdisease.
- Itsmeasurementisbasedondeterminingthevolumeofplasmafromwhichasubstanceisremoved by glomerular filtration during its passage through the kidney. In other words, the clearance ofthat substance, Clearance = (U x V) / P Where U = urinary concentration of X; V = rate of urine formation (ml/min); P = plasma concentration ofX.

Creatinine Clearance

- It is often used as a rough measurement of GFR, with a timed urine collection (often 24hrs).
- And a blood sample taken to measure plasma creatinine during that timeperiod.
- It is limited by problems of accurate urine collection and tends to overestimate theGFR.

Estimated GFR

- The plasma creatinine concentration (alone) is only a very rough guide to renalfunction.
- Creatinine is produced by the muscles at a relatively constant level by thebody.
- So, the plasma concentration therefore depends on the rate of excretion by thekidneys.
- Levels are affected by age, gender, and ethnic group, and muscle bulk, ingestionof cooked meat, malnutrition and after use of somedrugs.

Glomerular filtration rate (GFR)

- This is the most frequent test of renal function.
- GFR varies as a function of normal physiology as well asdisease.
- Its measurement is based on determining the volume of plasma from which a substanceis removed by glomerular filtration during its passage through thekidney.
- In other words, the clearance of that substance, Clearance = (U x V) / P Where U = urinary concentration of X; V = rate of urine formation (ml/min); P = plasma concentration ofX.

Creatinine Clearance

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- And a blood sample taken to measure plasma creatinine during that timeperiod.
- It is limited by problems of accurate urine collection and tends to overestimate theGFR.
- Levels are affected by age, gender, ethnic group, muscle bulk, ingestion of cookedmeat, malnutrition and after use of somedrugs.

Creatinine Clearance test

- This test evaluates how efficiently the kidneys clear a substance called creatinine from theblood.
- Creatinine clearance test is very specific measurement of kidney function and often used asa rough measurement ofGFR.
- The test is performed on a timed urine specimen— a cumulative sample collected over a twoto 24-hourperiod.
- Determination of the blood creatinine level is also required to calculate the urineclearance.

Urea Clearance test

- Urea is a waste product that is created by protein metabolism and excreted in theurine.
- The urea clearance test requires a blood sample to measure the amount of urea in the bloodstream andtwourinespecimens,collectedonehourapart,todeterminetheamountofureathatisfiltered, or cleared, by the kidneys into theurine.

Urine Osmolality test

- Urine osmolality is a measurement of the number of dissolved particles inurine.
- It is a more precise measurement than specific gravity for evaluating the ability of the kidneysto concentrate or dilute theurine.
- Kidneys that are functioning normally will excrete more water into the urine as fluid intakeis increased, diluting theurine.
- If fluid intake is decreased, the kidneys excrete less water and the urine becomesmore concentrated.

- The test may be done on a urine sample collected first thing in the morning, on multiple timed samples, or on a cumulative sample collected over a 24-hourperiod.
- The patient will typically be prescribed a high-protein diet for several days before the test and be asked to drink no fluids the night before thetest.

Urine Protein test

- Healthy kidneys filter all proteins from the bloodstream and then reabsorb them, allowingno protein, or only slight amounts of protein, into theurine.
- The persistent presence of significant amounts of protein in the urine, then, is an important indicator of kidneydisease.
- A positive screening test for protein (included in a routine urinalysis) on a random urine sample is usuallyfollowedupwithatestona24-hoururinesamplethatmorepreciselymeasuresthequantity ofprotein.

Other blood tests

Measurement of the blood levels of other elements regulated in part by the kidneys can also be useful in evaluating kidney function.

These include- BUN, Creatinine, Sodium, Potassium, Chloride, Bicarbonate, Calcium, Magnesium, Phosphorus, Protein, Uric acid, Glucose etc.

Exercise-10

Liver Function Tests

Definition

- Liver function tests, or LFTs, include
  - Tests for bilirubin, a breakdown product of hemoglobin,and
  - Test for ammonia, a protein byproduct that is normally converted into urea by the liverbefore being excreted by thekidneys.
  - Tests to measure levels of several enzymes, which help metabolizing othersubstances.
  - Enzymes that are often measured in LFTs include GGT, ALT (or SGPT) AST (or SGOT)and ALP.
  - LFTs also may include prothrombin time (PT), a measure of how long it takes for the bloodto clot.

Description

- The liver is one of the most important organs in the body. As the body's "chemical factory," it regulatesthelevelsofmostofthemainbloodchemicals.Itactswiththekidneystoclear theblood ofdrugsandtoxicsubstances.Thelivermetabolizestheseproducts,alterstheirchemicalstructure, makes them water soluble, and excretes them in bile. Liver function tests are used to determine if theliverhasbeendamagedoritsfunctionimpaired.Elevationsofcertainliverenzymesinrelation toothersaidsinthatdetermination.Forexample,aminotransferases(whichincludeALTandAST) arenotablyelevatedinliverdamagecausedbylivercelldisease(hepatocellulardisease).However, in intra-hepatic obstructive disease—which may be caused by some drugs or biliary cirrhosis— the alkaline phosphatases are mostabnormal.

Functions of Liver

- Detoxification ofdrugs
- Bile pigmentmetabolism
- Carbohydrate, fat, protein metabolisms
- Secretion of serumproteins
- Production of coagulationfactors
- Ureasynthesis
- Storage of vitaminsB_{12}

REGULATION, SYNTHESIS AND SECRETIONS

Clinically Important Serum Enzymes

Alanine Aminotransferase (ALT):

- It is an enzyme necessary for energy production. It is present in a number of tissues,including the liver, heart, and skeletal muscles, but is found in the highest concentration in theliver.
- Because of this, it is used in conjunction with other liver enzymes to detect liverdisease, especially hepatitis or cirrhosis withoutjaundice.
- Additionally, in conjunction with the aspartate aminotransferase test (AST), it helpsto distinguish between heart damage and liver tissuedamage.

Aspartate Aminotransferase (AST) :

- It also known as SGOT. Elevated in liver and heart disease. In liver disease, the AST increase is usually less than the ALT increase. However, in liver disease caused by alcohol use, the AST increase may be two or three times greater than the ALTincrease.

Alkaline Phosphatase (ALP)

- Its levels usually include two similar enzymes (isoenzymes) that mainly come from the liverand bone and from the placenta in pregnantwomen.
- Increased in liver disorders bone disorders**.**

Gamma-glutamylTransferase(GGT):

- Sometimes called gamma-glutamyltranspeptidase(GGPT)
- It is an enzyme that is compared with ALP levels to distinguish between skeletal diseaseand liverdisease.
- Because GGT is not increased in bone disorders, as is ALP, a normal GGT with an elevatedALP would indicate bonedisease.
- Conversely, because the GGT is more specifically related to the liver, an elevated GGT withan elevated ALP would strengthen the diagnosis of liver or bile-ductdisease.

Bilirubin

- Bilirubin, a breakdown product of hemoglobin, is the predominant pigment in a substance produced by the liver called bile. Excess bilirubin causes yellowing of body tissues(jaundice).
- There are two tests for bilirubin: direct-reacting (conjugated) and indirect-reacting (unconjugated). Differentiating between the two is importantdiagnostically.
- Elevated levels of indirect bilirubin are usually caused by liver cell dysfunction (e.g. hepatitis), Whileelevationsofdirectbilirubintypicallyresultfromobstructivejaundice.Theobstructionmay be either within the liver (intra-hepatic) or a source outside the liver (e.g. gallstones or a tumor blocking the bileducts).

- Bilirubin measurements are valuable in newborns, as highly elevated levels ofunconjugated bilirubin can accumulate in the brain, causing irreparabledamage.

Ammonia

- Analysis of blood ammonia aids in the diagnosis of severe liver diseases and helps to monitorthe course of thesediseases.
- Ammonia levels are also helpful in the diagnosis and treatment of hepatic encephalopathy, a serious brain condition caused by the accumulated toxins that result from liver disease and liver failure.

Glucose

- The liver plays a major role in maintaining concentrations of glucose, by storing or releasing glucose asneeded.

Proteins

- Most blood proteins (except for antibodies) are synthesized and secreted by theliver.
- One of the most abundant serum proteins isalbumin.
- Impaired liver function that results in decreased amounts of serum albumin may lead toedema, swelling due to fluid accumulation in thetissues.
- The liver also produces most of the proteins responsible for blood clotting, called coagulationor clottingfactors.
- If the blood cannot clot normally due to a decrease in the production of these factors,excessive bleeding mayresult.

Bile

- Bile is a greenish fluid synthesized by hepatocytes and secreted into biliaryducts.
- It then leaves the liver to be temporarily stored in the gallbladder before emptying into thesmall intestine.
- The major components of bile include cholesterol, phospholipids, bilirubin (a metabolite ofred blood cell hemoglobin), and bilesalts.
- Importantly, bile salts act as "detergents" that aid in the digestion and absorption of dietaryfats.
- Liver damage or obstruction of a bile duct (e.g., gallstone) can lead to cholestasis,steatorrhea (Fat in faeces) andjaundice.

Lipids

- Cholesterol, a type of lipid, is a substance found in cell membranes that helps maintain the physical integrity ofcells.
- The liver synthesizes cholesterol, which is then packaged and distributed to the body to besued or excreted into bile for removal from thebody.

- Increased cholesterol concentrations in bile may predispose to gallstoneformation.
- The liver also synthesizes lipoproteins, which are made up of cholesterol,triglycerides (containing fatty acids), phospholipids, andproteins.
- Lipoproteins circulate in the blood and shuttle cholesterol and fatty acids (an energysource) between the liver and bodytissues.
- Most liver diseases do not significantly affect serum lipid levels, with the exception of cholestatic diseases, which may be associated with increasedlevels.

STORAGE AND CLEARANCE

Storage

- The liver is designed to store important substances such as glucose (in the form ofglycogen).
- The liver also stores fat-soluble vitamins (vitamins A, D, E and K), folate, vitamin B 12 ,and minerals such as copper and iron.
- However,excessiveaccumulationofcertainsubstancescanbeharmful.Eg.PatientswithWilson's diseasecannotsecretecopperintobilenormallyandusuallyhavealowlevelofthecopper-binding proteinceruloplasmin.
- Retained copper accumulates in the liver leading to cirrhosis and neuropsychiatric symptoms(in CNS).

Purification, Transformation and Clearance

- The liver removes toxins and ammonia from the blood and then breaks them down ortransforms them into less harmfulcompounds.
- In addition, the liver metabolizes most hormones and ingested drugs to either more or lessactive product.
- **Ammonia**
  - The liver converts ammonia to urea, which is excreted into the urine by thekidneys.
  - In the presence of severe liver disease, ammonia accumulates in the blood because of both decreased blood clearance and decreased ability to formurea.
  - Elevated ammonia levels can be toxic, especially to the brain, and may play a role inthe development of hepaticencephalopathy.
- **Bilirubin**
  - Bilirubin is a yellow pigment formed as a breakdown product of red blood cellhemoglobin.
  - The spleen, which destroys old red cells, releases "unconjugated" bilirubin into the blood,where it circulates in the blood bound toalbumin.

- The liver efficiently takes up bilirubin and chemically modifies it to "conjugated," or water-soluble, bilirubin that can be excreted intobile.
- Increased production or decreased clearance of bilirubin results in jaundice, anyellow pigmentation of the skin and eyes from bilirubinaccumulation.

- **Hormones**
- Since the liver plays important roles in hormonal modification and inactivation, chronic liver disease may cause hormonal imbalances.Foreg. The masculinizing hormone testosterone and the feminizing hormone estrogen are metabolized and inactivated by theliver.
- Men with cirrhosis, and who abuse alcohol, have increased circulating estrogens relativeto testosterone derivatives, which may lead to bodyfeminization.

- **Drugs**
- Nearly all drugs are modified or degraded in the liver, particularly; oral drugs are absorbed by the gut and transported via the portal circulation to theliver.
- In the liver, drugs on metabolism, are modified, activated or inactivated before they enterthe systemic circulation, or they may be leftunchanged.
- Alcohol is primarily metabolized by the liver, and accumulation of its products can lead tocell injury anddeath.
- In patients with liver disease, drug detoxification and excretion may be dangerouslyaltered.
- This results in drug concentrations that are too low or too high or the production of toxic drug metabolites.

- **Toxins**
- The liver is generally responsible for detoxifying chemical agents and poisons, whetheringested orinhaled.
- Pre-existing liver disease may inhibit or alter detoxification processes and thus increase the toxic effects of theseagents.
- Additionally, exposure to chemicals or toxins may directly affect the liver, ranging frommild dysfunction to severe and life-threateningdamage.

Test based on protein metabolism

- Total Proteins Albumin andGlobulins
- The levels of plasma total proteins may be expressed asg/dL
- Measurement of total protein concentration is of limited clinicalvalue.

- Prothrombin time
- Prothrombin is increased in liver disease..

- Turbidity/Flocculationtests
  - Thymol turbidity test: The degree of turbidity produced when serum is mixed with abuffered solution of thymol. Sera from patients with liver disease tend to produce moreturbidity.
  - Zinc Sulphate turbidity test: Addition of normal serum to a solution of Zinc Sulphateproduces turbidity. Sera with higher concentration of gamma globulin produce moreturbidity.

Excretory functions Tests

- The Bromsulphthalein (BSP)test
  - The ability of the liver to remove a dye form the blood is determined. It is an indication of the efficiency of the liver to remove other substances from the blood, which are excreted in the bile. Bromsulphthalein and indocyanin green are the most frequently used dyes to assess the liver function. The test is carried out by the intravenous injection of these dyes and drawl of blood samples and estimation of the concentration of the dye in the plasma. Delayed clearance indicates the abnormal function ofliver.
- Hippuric acid synthesistest
  - This test is based on the ability of the liver to conjugate benzoic acid with glycine toform hippuric acid, which is excreted inurine.

ESTIMATION OF SERUM ALKALINE PHOSPHATASE (p-NPP method – Kinetic mode)

Principle:

- Alkaline phosphatase (ALP) hydrolyzes p-nitrophenyl phosphate (p-NPP) into p-nitrophenol and inorganic phosphate at pH 10.0. The p-nitrophenol is a yellow compound, which absorbs light at 405 nm. The rate of increase in absorbance at 405 nm is proportional to alkaline phosphatase activity inspecimen.

$$\text{p-nitrophenyl phosphate} + H_2O \xrightarrow{\text{ALP}} \text{p-nitrophenol} + \text{inorganic phosphate}$$

Reagents:

- Reagent 1 (Diethonalamine buffer, Magnesiumchloride)
- Reagent 2 (p-nitrophenylphosphate)

Preparation of working reagent:

- Mix four Volumes of Reagent 1 with one volume of Reagent 2. This working reagent isstable for 30 days at 2 – 8 ^{0}C.

Procedure:

- Mark one clean test tube as(T):

S. N.	Reagents	T
1.	Working Reagent	1000 m l
2.	Serum	20 m l
3.	Distilled Water	2000 m l

- Mix well and incubate for 1 minute at 37^{o}C. Read the change in absorbance per minute (Δ OD/ min.) during 3 minutes at 405 nm, setting zero withD.W.

Calculation:

- Serum ALP activity (U/L) = (Δ OD / min.) x 2750 x3

Result:

- The ALP activity of the given serum =U/L.

Clinical Significance:

- SerumALPestimationisusefulindiagnosisofhepatobiliarydiseasesandalsodiseasesassociated withincreasedosteoblasticactivity.Itiselevatedinosteomalaciaandrickets.Veryhighlevelsare found in patients with bonecancer.
- Elevated levels are also found in intra-hepatic obstruction due to stone or spasm and also in extra-hepatic obstruction (cholestasis) where the levels are stillhigher.

Appendix- 1

ATOMIC WEIGHTS OF SOME COMMON ELEMENTS

Elements	Symbols	Atomic number	Atomic weight
Aluminum	Al	13	26.98
Calcium	Ca	20	40.08
Carbon	C	6	12.01
Chlorine	Cl	17	35.453
Chromium	Cr	24	51.99
Cobalt	Co	27	58.93
Copper	Cu	29	63.54
Hydrogen	H	1	1.008
Iodine	I	53	126.90
Iron	Fe	26	55.84
Magnesium	Mg	12	24.30
Manganese	Mn	25	54.93
Mercury	Hg	80	200.59
Nitrogen	N	7	14.00
Oxygen	O	8	15.99
Phosphorus	P	15	30.97
Potassium	K	19	39.09
Silver	Ag	47	107.86
Sodium	Na	11	22.99
Sulphur	S	16	32.06
Zinc	Zn	30	65.38

Appendix- II

STRENGTH OF AQUOUS SOLUTION OF SOME CONCENTRATED ACIDS AND BASES

	Mol. Formula	Mol. Mass	Approximate		Volume required (ml) to make 1 M Solution
			% of solute by weight	Specific gravity	
Nitric Acid	HNO_3	63.0	70	1.18	63
Hydrochloric Acid	HCI	36.5	35	1.42	36.5
Sulphuric Acid	H_2So_4	98.0	96	1.84	56
Acetic Acid	CH_3CooH	60.0	100.0	1.05	58
Phosphoric Acid	H_3Po_4	98	>85	1.70	68
Sodium Hydroxide	NaoH	40	50	1.48	53

Appendix- III

WEIGHTS OF SUBSTANCES REQUIRED TO PREPARE ONE LITRE OF SOLUTION OF A PARTICULAR NORMAL

Substance	Mol. Mass	Eq. Mass	Quantity in grams for solution of strength	
			N/10	N/20
Oxalic Acid	126.08	61.04	6.304	3.152
Sodium Carbonate	106.00	53.00	5.3	2.65
Calcium Carbonate	100.00	50.00	5.00	2.50
Sodium Hydroxide	40.00	40.00	4.00	2.00
Sodium Bicarbonate	84.00	84.00	8.40	4.20
Potassium Permagnate	153.03	31.60	3.610	1.580

Appendix- IV

CLINICAL BIOCHEMISTRY-REFERENCE VALUE

Laboratory test	Dog	Cat	Horse	Cow	Pig	Sheep
Alkaline phoshatase (IU/L)	20-150	10.0-80	143-395	90-170	26-362	68-387
ALT(IU/L)	10.0-88.0	10.0-80	34-113	14-38	32-84	60-84
Amylase (IU/L)	300-2000	500-1800	35-100	126-250		
AST(IU/L)	10.0.88.0	10.0.80	226-366	78-132	9-113	98-278
Bile acids(umol/l)	<10	<5	<15			
Bile acids post feed (umol/L)	<25	<15				
Billirubin, total (mg/dl)	0.1.0.06	01-0.6	0-2.0	0.10.5	0-02	0-0.4
Calcium(mg/dl)	8.611.2	8.0-10.7	11.2-13.6	9.7-12.4	8-12	10.4-13
Chloride (mmol/L)	105-115	117-123				
Cholesterol (mg/dl)	125-270	90-205				
Cobalamin (vitamin B_{12} (ng/L)	300-800	200-1680				
Cortisol, resting (mg/dL)	1.0-4.0	1.01.3				
Creatinine kinase (IU/L)	20-200	50-450	86-140	66-120		
Creatinine (mg/dL)	0.5-1.5	0.8-1.8	1.2-1.9	1-2.0	1.0-2.7	1.2-1.9
Fibrinogen (mg/dL)	125-300	100-400	200-400	200-500	200-400	100-500
Folate (mg/L)	7.5-17.5	13.4-38				
GGT(IU/L)	1.0-10.0	1.0-10	4-13.4	11-24		
Glucose (mg/dL)	60-110	70-150	75.115	45-75	65-95	50-80
Iron (mg/L)	94-122	68-215	73-140	57-162	91-199	166-222
LDH (IU/L)	50-495	75-490	162-412	8-302	96-160	60-111
Lipase (IU/L)	25-750	25-375				

Phosphorus (mg/dl)	2.2-55	1.8-6.4	3.1-5.6	5.6-605	5.3-9.6	5.0-7.3
Potassium (mEq/L)	3.7-5.8	3.8-4.5	2.4-4.7	3.9-5.8	4.9-7.1	4.0-6.0
Protein (g/dL)	5.4-7.7	5.4-7.8	5.2-7.9	6.7-7.5	7.0-8.9	6.0-7.9
Albumin (g/dL)	2.3-3.8	2.1-3.9	2.6-3.7	3.0-3.6	1.9-3.3	2.4-3.9
Globulin (g/dL)	2.3-5.2	1.5-5.7	2.6-4.0	3.0-3.5	5.3-6.4	3.5-5.7
Sodium (mEq/L)	141-153	147-156	132-146	132-152	139-152	136-154
Sorbitol dehydrogenase (IU/L)	2.9-8.2	3.9-7.7	1.9-5.8	4.3-15.3	1-6	6-28
T3 (ng/dL)	75.200	60.200	31-158	41-170		
T4 (mg/dL)	1.0-4.0	1.5-5.0	1.0-2.4	3.6-8.9		
T4 free (ng/dL)	0.7-3.3					
TLI (mg/L)	5.35					
Urea nitrogen (mg/dl)	12.0-25.0	10.0-30.0	10.0-24	20-30	8.24	18-31

Appendix- V

URINE VALUES

S. No.	Parameters	Horse	Cattle	Sheep	Pig	Dog	Cat
1	Volume	3-10	6.25	0-5.2	2-6	0.04-2	0.075-0.2
2	L/day	1.035	1.015	1.030	1.015	1.025	0.2
3	Specific gravity mean	1.020-1.050	1.005-1040	1.020-1.040	1.010-1.030	1.018-1.045	1.030 1.020-1.040
4	pH Range	7.0-8.5	8.5	7.0-8.5	6.0-8.5	5.2-6.8	
5	Glucose	Nil	Nil/trace	Nil/trace	Nil	Nil/trace	Nil
6	Protein	Nil	-	Trace	Nil/trace	Trace	Nil/trace
7	Billrubin	-	Trace	-	-	-	-
8	Ketones	+	Trace	Variable	Nil	-	Nil
9	Indican	-	+	-	-	-	-
10	Magnesium		<0.8	-	-	-	-

+ Present, - Absent, a few epithelial cells, RBC and crystals can be found in normal urine.